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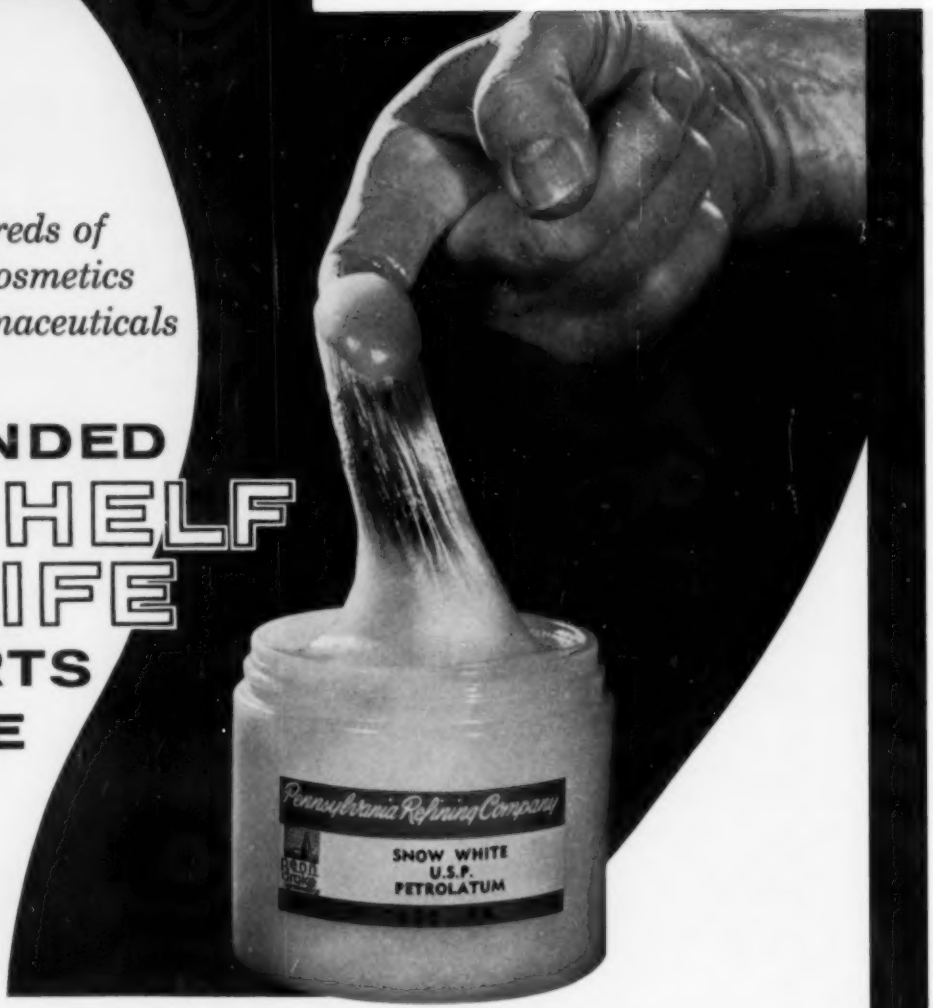

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Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1959 Award, *nominations must be received on or before March 31, 1959.*

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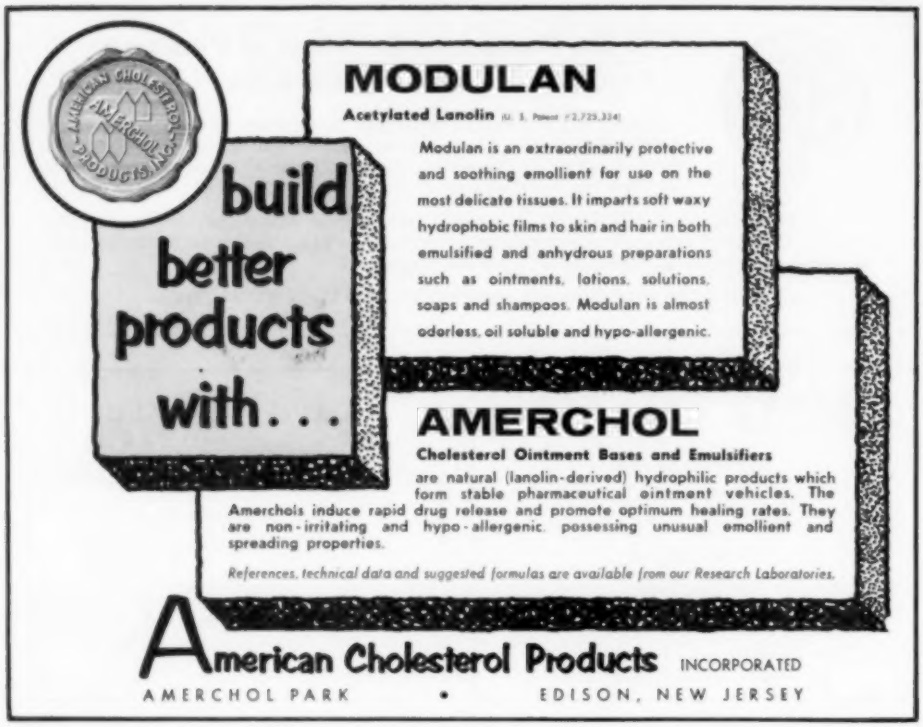
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The next annual meeting of the American Pharmaceutical Association will be held in Cincinnati, Ohio, August 16 to 21, 1959. Titles and abstracts (not to exceed 200 words) of papers must be submitted to the appropriate Section secretary not later than June 1, 1959, or preferably earlier, in order to assure a place on any Section program.

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VOLUME XLVIII FEBRUARY 1959 NUMBER 2

A Kinetic Study of the Specific Hydrogen Ion Catalyzed Solvolysis of Chloramphenicol in Water-Propylene Glycol Systems*

By ARNOLD D. MARCUS† and ANTHONY J. TARASZKA

The specific hydrogen ion catalyzed degradation of chloramphenicol in solutions containing up to 50 per cent (v/v) propylene glycol is qualitatively similar to degradation in purely aqueous systems. The reaction remains pseudo-first order with respect to chloramphenicol and the direct dependence of the rate upon acid concentration is maintained. The contributions of a water reaction at low acid concentrations is still evident in 10 per cent glycol systems but not in 30 or 50 per cent glycol. If the previously postulated mechanism for this reaction is correct, the present data are in conflict with the requirements of the Bronsted-Christiansen-Scatchard equation for a reaction between ions of like sign. In the present study a decrease in dielectric constant resulted in an increase rather than a decrease in the rate of the reaction. The inverse relationship between rate and dielectric constant is rationalized by assuming the reaction to be of the ion-dipole rather than of the ion-ion type. This assumption appears to receive support from the qualitative agreement between the data and the requirements of the Amis equation and a mechanism is offered to account for the postulated ion-dipole reaction. The activation energies for the reactions in 10, 30, and 50 per cent propylene glycol are 21.0, 20.5, and 20.0 kilocalorie/mole, respectively. The direct relationship between activation energy and dielectric constant agrees with the theoretical requirements for a cation-dipole reaction.

THIS INVESTIGATION was undertaken in an attempt to study the specific solvent or other effects of propylene glycol upon the rate of hydrogen ion catalyzed degradation of chloramphenicol in water-propylene glycol solutions. A major objective of this study was the critical and, if possible, quantitative evaluation of the effect of increasing glycol concentrations upon the rate of hydrolysis or other solvolytic cleavage, if any. Such a study appeared to be of particular importance in view of the widespread use of mixed solvents in pharmaceuticals and the relative lack of information concerning the effects of such solvent systems upon the stability of active ingredients.

The hydrolytic, or other solvolytic, decomposition of pharmaceuticals is by no means a new problem. Drugs of such diverse chemical constitution as procaine, aluminum acetate, naphazoline, and chloramphenicol are all subject to hydrolytic breakdown (1–4) and these liabilities are often important in formulation procedures. In many instances solvolysis may be inhibited, or avoided entirely, by a variety of remedies including judicious maintenance of pH (1, 2), "tying up" the labile species in the form of a stable complex (5), the use of an insoluble form of the active ingredient (6), or by careful control of buffer capacity and constituents (7).

Despite the apparent universality of the above procedures, it is often found that their application fails to achieve the desired resistance to solvolysis.

* Received May 3, 1957, from College of Pharmacy, Rutgers University, Newark 4, N. J.
Presented to the Scientific Section, A. PH. A., New York Meeting, May, 1957.
This study was supported, in part, with funds supplied by the Research Council of Rutgers University.
Portions of this paper are adapted from the prize-winning manuscript submitted by Anthony J. Taraszka in the Eastern Region, 1956, Lunsford Richardson Pharmacy Award competition.
† Present address: Merck Sharp and Dohme Research Laboratories, West Point, Pa.

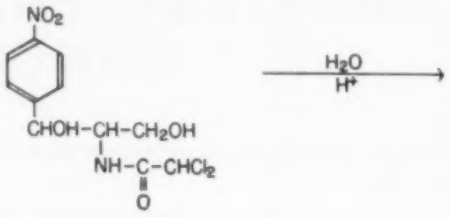
We often find, therefore, that the problem is attacked by a partial or complete change in the solvent system. It has been recently reported (8), for example, that the stability of pentobarbital in solution is materially enhanced by the use of a polyethylene glycol solvent system. Similar reasoning has been successfully applied in the official monographs on tribromethanol solution, isofluophate solution, nitrofurazone solution (9–11), and other preparations.

It is probably as a result of these and similar successes that there is a generally held opinion that the use of nonaqueous solvents to replace all, or a portion of, the water in a solution is some sort of panacea. Such an assumption is largely erroneous and probably results from a lack of appreciation of the ability of nonaqueous solvents, especially those which are hydroxylic, to participate in, or otherwise influence, solvolytic reactions. Many of the claims which have been advanced in this area are either without sound foundation in experimental fact or are the results of purely superficial evidence. Water may have been branded a villain too often without good cause.

It is probable that this situation has resulted, at least in part, from the scarcity of reliable chemical kinetic studies regarding the rates of degradation of pharmaceuticals. An accurate evaluation of the specific or other contributions of a second solvent is difficult, if accurate and precise studies of degradation in water have not been carried out.

As part of a project concerning the effects of nonaqueous solvents upon the stability of pharmaceuticals, it was decided to study, initially, propylene glycol. This solvent is finding increasing use and is of such a chemical constitution that it might very possibly participate in solvolytic reactions. Since only a few drugs have been evaluated with respect to their behavior in aqueous systems, it was decided that it would be most prudent to choose for this study, a drug which decomposed solvolytically in acid systems, thus eliminating the need for buffer systems and pH measurements, both of which may have little significance in mixed solvents. Inasmuch as the response of chloramphenicol, in purely aqueous systems, to temperature and pH has been almost completely evaluated, this antibiotic was chosen for investigation.

Since it was possible that the inclusion of propylene glycol in the reaction media might alter the order and/or character of the degradative reactions or affect the formation or interaction of ionic species, the reactions were carried out in water-propylene glycol systems containing up to 50 per cent (v/v) glycol. The data obtained were analyzed with a view toward determining, if possible, the degrees of both hydrolytic and non-hydrolytic solvolysis (Figs. 1 and 2) as well as the influence of any electrostatic effects.



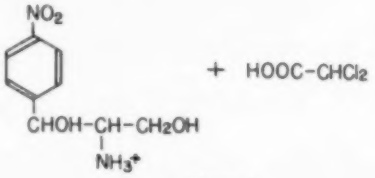
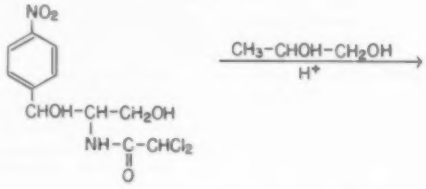


Figure 1.



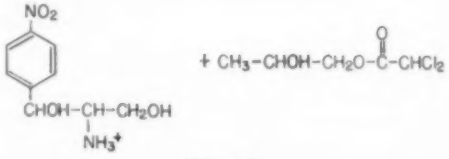


Figure 2.

EXPERIMENTAL

Reagents.—Other than the chloramphenicol and U. S. P. grade propylene glycol, all chemicals were reagent grade.

Method of Degradation.—The general experimental procedure of Higuchi, *et al.* (7, 12), was employed to effect all degradations. The glycol-perchloric acid solutions were prepared by adding the glycol to the standard solution of perchloric acid, dropwise, through a 25-ml. buret. This procedure minimized any errors which might have resulted from the viscosity of the glycol.

Analytical Procedure.—Partition chromatographic elution of residual undegraded chloramphenicol followed by spectrophotometric determination of the antibiotic as reported by Higuchi, *et al.* (13),

was employed throughout, with the following modifications: The mobile phase was changed from 10 to 15% (v/v) ethyl acetate in chloroform and all spectrophotometric measurements were made at 272 mμ, the peak for chloramphenicol in this solvent. In addition, the samples of reaction mixture containing 50% glycol were diluted with an equal volume of purified water prior to separation.

DISCUSSION OF THE EXPERIMENTAL RESULTS

Nature of the Acid Catalyzed Reaction.—It is evident from Fig. 3 that the addition of propylene glycol to the reaction systems in no way affects the pseudo-first order character of the overall degradative reaction. While this evidence is not conclusive, the nature of these plots, along with the previously demonstrated behavior of chloramphenicol in purely aqueous systems, overwhelmingly indicates that here, too, solvolysis is the major degradative pathway. For this reason it will be implicitly assumed in the balance of the discussion that solvolysis represents the only significant route of degradation. It should be noted, however, that these plots cannot, except indirectly, indicate the degree to which propylene glycol influences the rate of degradation. The data, plots, and discussion to follow may serve to illuminate this area.

Previous studies (7, 12) concerning the degradation of chloramphenicol have shown that in aqueous systems below pH 7, three major types of reactions are responsible for degradation; general acid-base catalyzed hydrolysis, specific hydrogen ion catalyzed hydrolysis, and an uncatalyzed or "water" reaction. In highly acid systems, however, the rate of degradation depends only upon the temperature and acid concentration. In solutions where the concentration of acid is low (*ca.* 0.025 *M*), however, the total rate represents a summation of the hydrogen ion catalyzed reaction and the "water" reaction. As a result, the rate of hydrolysis of chloramphenicol is significant even at "zero" hydrogen ion concentration.

This behavior, plus the experimentally established increase in the solubility of chloramphenicol with increasing hydrogen ion concentration, led Higuchi and Marcus (12) to postulate that the hydrogen ion and the uncatalyzed solvolyses are associated with different substrates. These authors said that the substrate in the acid catalyzed reaction is a monoprotonated chloramphenicol with a positive charge on the substrate, whereas the substrate in the "water" reaction is chloramphenicol itself.

Figures 4, 5, and 6 show that the linear relationship between rate of degradation and acid concentration (above 0.025 *M*) is unaffected by including 10, 30, or 50% propylene glycol in the reaction mixtures. There is, however, a notable difference between the plots in Fig. 4 and those in Figs. 5 and 6, even at low concentrations of acid. If the lines in Fig. 4 were drawn so as to pass through all of the experimentally determined points, they would not pass through the origin (as drawn) but would curve or level off to intersect the ordinate significantly above the origin. In light of the preceding discussion concerning the

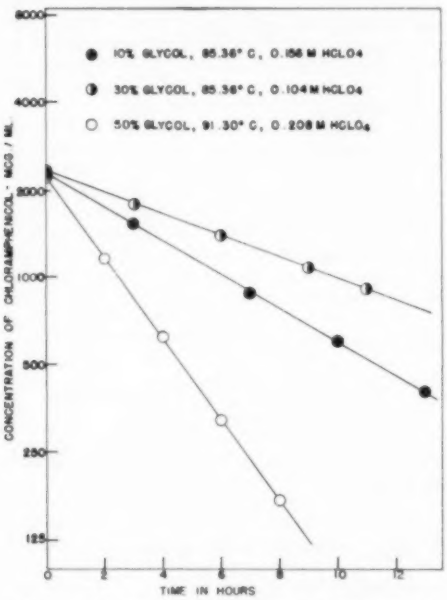

10% GLYCOL, 85.36° C, 0.156 M HCLO4
30% GLYCOL, 85.36° C, 0.104 M HCLO4
50% GLYCOL, 91.30° C, 0.208 M HCLO4
CONCENTRATION OF CHLORAMPHENICOL - MCG / ML
TIME IN HOURS

Fig. 3.—Plots showing the rate of hydrogen ion catalyzed hydrolysis of chloramphenicol to remain first order with respect to the antibiotic under a variety of experimental conditions.

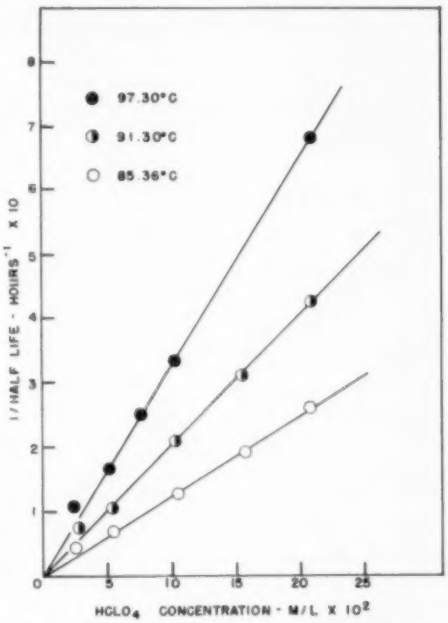


Fig. 4.—Plots showing the direct relationship between the rate of acid catalyzed degradation of chloramphenicol and acid concentration in 10% (v/v) propylene glycol. As noted in the text, the departures from linearity at low concentrations of acid may be ascribed to the contributions of the "water" reaction.

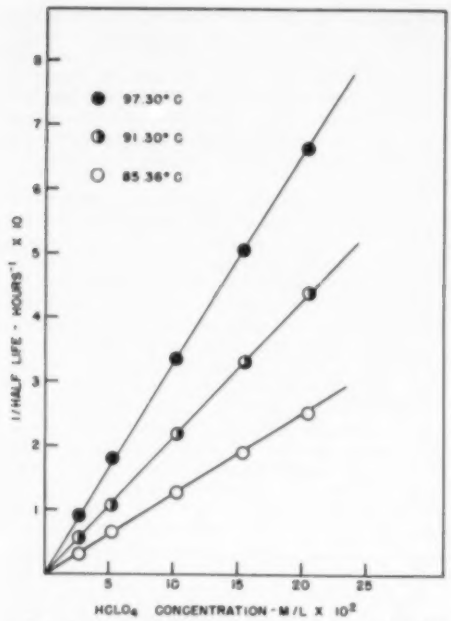


Fig. 5.—Plots similar to those in Fig. 4 but for 30% (v/v) propylene glycol. The lack of any noticeable deviations from linearity at low concentrations of acid indicate that the "water" reaction is either absent entirely or that its contributions are negligible.

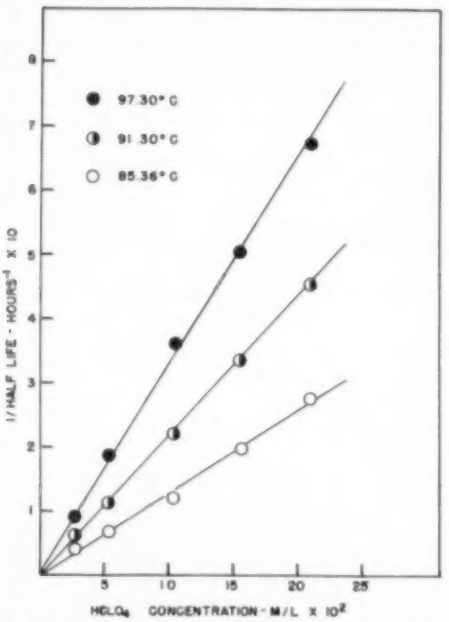


Fig. 6.—Plots similar to those in Fig. 5 but for 50% (v/v) propylene glycol.

dual degradative pathways, it is clear that in the solutions containing only 10% propylene glycol, the "water" reaction still contributes significantly at low hydrogen ion concentrations.

In Figs. 5 and 6, however, a much different picture is presented. In these plots all of the experimentally determined points, including those obtained at 0.026 *M* acid, fall directly on the lines drawn to pass through the origin. There is no discernible evidence of any levelling off. It is, therefore, possible to conclude that in reaction systems containing 30 or 50% propylene glycol and 0.026 *M* acid, the uncatalyzed reaction is either absent entirely or else plays so small a role as to be beyond the power of resolution of the analytical procedures employed. One thing appears certain, in reaction systems containing higher concentrations of the glycol, the "water" reaction is substantially suppressed either as a result of simple decrease in the relative concentration of water, or because the glycol in some manner favors the mono-protonated form of the substrate. Mono-protonated chloramphenicol is, of course, virtually unaffected by the "water" reaction.

Although the preceding discussion and supporting figures indicate that at low hydrogen ion concentrations the addition of propylene glycol tends to reduce the overall rate of degradation, a quite different view is gained upon inspection of the plots in Figs. 4, 5, and 6 at higher acid concentrations. While the qualitative character of the specific hydrogen ion catalyzed solvolysis of chloramphenicol is essentially unaffected by the inclusion of glycol in the reaction systems, a change in qualitative character was not actually anticipated. It was expected, however, that the quantitative picture would be quite different. In line with generally held opinions, we assumed that the rate of degradation would decrease significantly with increasing concentrations of propylene glycol. This assumption was supported, from a theoretical standpoint, by the requirements of the Bronsted-Christiansen-Scatchard equation (14) for reactions between ions of like sign.

We were, therefore, surprised to find that the inclusion of propylene glycol did not result in a decrease in the rate of the hydrogen ion catalyzed reaction. Instead, it became apparent that an increase in glycol concentration caused an increase in the catalytic activity of the hydrogen ion and, therefore, in the rate of the reaction. These small but definite increases are summarized in Tables I and II. It should also be noted that there is an apparent lack of any regular relationship between the rate of reaction and the concentration of propylene glycol.

DISCUSSION OF THE REACTION MECHANISM

It is possible to rationalize the increases in the rates of reaction with increasing concentrations of propylene glycol in several ways. It seemed possible that upon adding glycol to the reaction systems there would be a competition between water and the glycol, so that the systems would contain both hydronium ions and glycolated protons. If attack by a glycolated proton is facilitated by the newly increased possibilities for van der Waals' type interactions with the substrate, the increase in rate of degradation need not remain puzzling. This proposal

TABLE I.—CATALYTIC CONSTANTS FOR HYDROGEN ION CATALYZED REACTION IN THE VARIOUS SYSTEMS

Temp., °C.	Glycol, %	k_H L. moles^{-1} hours^{-1}
97.30	0	2.00[a]
97.30	10	2.18
97.30	30	2.21
97.30	50	2.22
91.30	0	1.223[a]
91.30	10	1.433
91.30	30	1.469
91.30	50	1.490
85.36	10	0.845
85.36	30	0.875
85.36	50	0.902

[a] This value obtained from the data of Higuchi and Marcus (12).

TABLE II.—THE EFFECT OF THE CONCENTRATION OF PROPYLENE GLYCOL UPON THE RATE OF SOLVOLYSIS OF CHLORAMPHENICOL

Temp., °C.	Glycol, %	Half Life-Hours[a]
97.30	0	3.47[b]
97.30	10	3.17
97.30	30	3.14
97.30	50	3.13
91.30	0	5.67[b]
91.30	10	4.84
91.30	30	4.72
91.30	50	4.65
85.36	0	8.54[b]
85.36	10	8.23
85.36	30	7.93
85.36	50	7.69

[a] Half lives in 0.1 *M* perchloric acid.
[b] This value obtained from the data of Higuchi and Marcus (12).

is seriously set back, however, by the fact that at higher concentrations of the glycol (30 and 50%) the increase in rate does not begin to compare with the increase in glycol concentration. Since the existence of any substantial concentrations of glycolated protons would demand the presence of substantial amounts of propylene glycol, the fact that the rate of degradation is almost independent of glycol concentration in the range 10 to 50% glycol led us to abandon pursuit of this possibility.

In the course of our attempts to explain the apparent departure from expected behavior, the possibility was advanced that a glycolated proton would be a stronger acid than the hydronium ion, thus achieving a greater catalytic effect. The same deficiencies which caused us to discard the possibility of enhancement of catalytic activity by some sort of van der Waals' type interaction seemed operative here, too, and this second possibility was also discounted.

If the mechanism proposed by Higuchi and Marcus (12) is correct, the data in Tables I and II are a source of serious concern. Even though the rate of reaction is almost independent of glycol concentration, there can be no doubt of continuing slight increases in rate. Inasmuch as the mechanism proposed requires a reaction between two ionic species bearing the same sign, the data in Tables I and II are in direct conflict with the requirements of the Bronsted-Christiansen-Scatchard equation (14). This expression requires a *decrease* in the rate of reaction between ions of like sign when the dielectric constant of the medium is lowered.[1] In the present study, however, the rate of reaction *increased* when the dielectric constant was lowered.

According to Amis (17) the rate of a reaction between a positively charged ion and a dipolar molecule should increase as the dielectric constant of the reaction medium is lowered. The expression derived by Amis also predicts that the relationship between rate and dielectric constant is not linear but falls off with succeeding decreases in dielectric constant. The data in Table II seem to be quite in line with these requirements and thus lend support to the postulation that the reaction might involve an ion and a dipole.

Because the evidence cited above can hardly be said to offer any rigorous support, a means was sought to provide additional evidence in favor of the postulation. Amis' equation may, for the limiting case of head-on approach of an ion to a dipolar molecule, be written

$$\ln k_D = \ln k_{D=\infty} + \frac{Zeu}{Dk_bTr^2}$$

where D is the dielectric constant of the medium, Ze is the charge on the ion, k_b is the Boltzmann gas constant, T the absolute temperature, and r is a measure of the closeness of the approach of the ion. According to this equation, when the reactants are a cation and a dipolar molecule, a plot of ln k *vs.* $1/D$ should yield a straight line with a positive slope. In order to see whether or not our data would fulfill this requirement, however, it was necessary to know the dielectric constants of our water-propylene glycol systems at the temperatures employed to effect degradation.

A search of the literature failed to reveal any information concerning the dielectric constants of water-propylene glycol mixtures. We were forced, therefore, to make certain approximations. We realize that our approach was arbitrary and that the values and the plots derived from them are either meaningless or, at best, open to serious question. We felt, however, that any clarification which would result might possibly offset the liabilities inherent in our approach.

The data of Akerlof (18) and calculations made using the constants given by him for glycerol and *n*-propanol, along with similar calculations for propylene glycol (16), indicated that, from 20 to 100°, the dielectric constant of propylene glycol is approximately the arithmetic mean of the dielectric constants of glycerol and *n*-propanol with a maximum deviation of 3%. Akerlof also shows that his expressions for the temperature dependencies of the dielectric constants of glycerol and *n*-propanol hold

[1] The dielectric constant of water through the temperature range employed in this study varied from 59.6 to 56.1 as calculated from the work of Malmberg and Maryott (15). The similar variation in the dielectric constant of propylene glycol is 21.2 to 19.8 as calculated from the data of Morgan and Yager (16). Naturally, an increase in the concentration of glycol results in an overall decrease in the dielectric constant of the reaction system. For purposes of comparison, the dielectric constants of water and propylene glycol at 20° are 80.1 and 32.0, respectively (15, 16).

for aqueous solutions of these two substances as well as for the pure materials. In view of the approximate relationship between the dielectric constants of pure *n*-propanol, glycerol, and propylene glycol, we have assumed a similar relationship to hold for aqueous solutions of the three organic solvents. The values thus arrived at for the dielectric constants of water-propylene glycol mixtures were applied to our data in an attempt to see if a plot of log k *vs.* $1/D$ would aid in interpreting our data.

Our plots were of the required slope and definitely linear in the region between 10 and 50% propylene glycol. The plots did not, however, include the points at zero glycol concentration. Although these plots did lend slight weight to the possibility that the reaction could be classified as of the ion-dipole type, the slopes of the straight line portions were so small that it did not seem possible to place any great deal of faith in the results. It appeared that some alteration in approach would be necessary to achieve plots more indicative of the applicability of the Amis equation.

The apparently very small effect of glycol concentration in the range 10 to 50% glycol led us to assume that the glycol did not exert any effect such as summarized in Fig. 2. It appeared possible that any influence being exerted by the glycol might be purely electrostatic in nature. It has already been noted that when the rate equation may be written

$$k_{obs.} = K_{H^+}[H_2O]$$

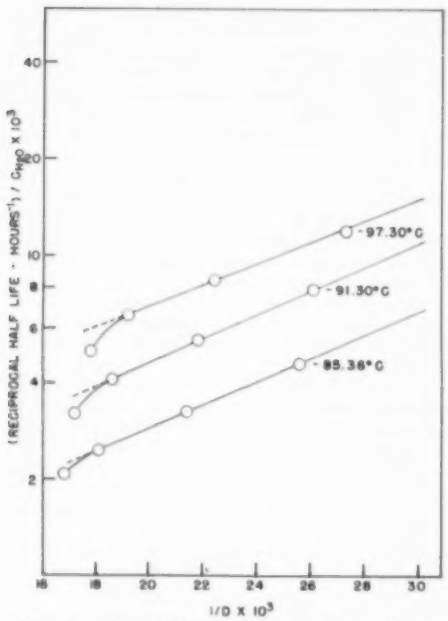
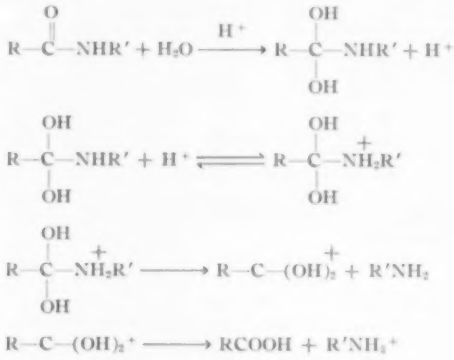


Fig. 7.—Plots indicating that our data for the reactions in 10, 30, and 50% (v/v) propylene glycol obey the requirements of the Amis equation for the effect of dielectric constant on the rate of a reaction between a cation and a dipolar molecule. The reciprocal half-lives were calculated from the data in Table II.

it is unrealistic to ignore the decrease in water concentration if the effect of the organic solvent is purely electrostatic (19). A better test of the obedience of our data to the requirements of the Amis equation would be, therefore, to plot log (reciprocal half-life)/C_{H_2O} as a function of $1/D$.[2] The results of these plots are shown in Fig. 7.

The magnitudes and the directions of the slopes in these plots do much to substantiate the thesis that the reaction studied involves an ion and a dipole. When viewed in terms of the means used to estimate the dielectric constants, the almost strict adherence of the plots, in the range of 10 to 50% propylene glycol, to the Amis equation is almost incredible. It should, therefore, be re-emphasized that any attempt to represent these plots as having much quantitative significance would be foolhardy.

Because of the degree of support in favor of the postulation of an ion-dipole reaction, we have attempted to inspect the mechanism proposed by Higuchi and Marcus in the hope of finding some indication that the postulated ion-dipole reaction does not represent either a "pipe dream" or a paradox. If that mechanism is rewritten to conform with the mechanism proposed by Bender and Ginger (20) for the acid catalyzed hydrolysis of amides, we could write



If the first step above can be regarded to explain the apparent increase in the solubility of chloramphenicol with increasing concentrations of acid as resulting, not from the formation of an ionic substrate but rather as due to the formation of the more polar R-C(OH)$_2$ — NHR′, the overall reaction need no longer be considered to be between two ions of like sign. Since the actual hydrogen ion catalyzed hydrolysis is dependent upon the second reaction, the dipole reaction may well be correct.

It may be objected that the first reaction cannot be regarded alone but represents just a first step in a series of reactions leading to eventual cleavage of the C—N bond. The experimental work showing an apparent increase in the solubility of chloramphenicol with increasing concentrations of acid was, however, carried out at 30°. At this temperature the rate of hydrolysis of chloramphenicol (due to the second reaction) is almost negligible. Under these

[2] Reciprocal half lives are related to the rate constants by the expression k = reciprocal half life × 0.693 for first order reactions.

circumstances it is not at all surprising that only the fast reaction (the first) would receive attention.

The second reaction above is clearly of the ion-dipole type. If the mechanism is correct, our data are no longer paradoxical but conform directly with theoretical requirements. The above mechanism also does much to explain the fact that, in purely aqueous systems, the activation energy for the hydrogen ion catalyzed reaction is 5 Kcal./mole less than for the "water" reaction (12).

Thus far nothing has been said about the nonlinearity of the plots in Fig. 7 in the range 0 to 10% glycol. The data and supporting plots do little to illuminate this area. Landskroener and Laidler (21) have noted "kinetic anomalies" in the region of 10% by weight of the organic component. It is possible that these departures from linearity represent just such an anomaly. We are unprepared to venture any explanation until this region has been explored in greater detail. We do find one hopeful note in the plots of Fig. 7. It appears that the magnitude of departure from linearity decreases with a decrease in temperature. It is possible that the decreased effect of temperature on dielectric constant plays some part in this progression toward linearity. It is also possible, however, that the effect of propylene glycol in the 0–10% region is due to a specific solvent effect and is not amenable to treatment from a standpoint of electrostatic theory alone.

It has been suggested that the postulated ion-dipole reaction could receive additional support if the parameter r in the Amis equation were evaluated and found to be reasonable, i.e., if it were found to be of the correct order of magnitude for a molecular radius. Such an evaluation would require a knowledge or an approximation of the dipole moment of the substrate. Inasmuch as this value is not known and an approximation from the moments of other *N*-substituted amides would still leave us with the problem of further approximating the influence of the proton already present on the substrate, we feel that little is to be gained by such an effort.

TEMPERATURE DEPENDENCIES

The Arrhenius-type plots in Fig. 8 indicate the effect of temperature on the overall rates of degradation in the several solvent systems. The energies of activation, as determined from the slopes of these plots are 21.0, 20.5, and 20.0 Kcal./mole in the 10, 30, and 50% glycol solutions, respectively. For purposes of comparison, the activation energy in pure water is 19.5 Kcal./mole (12).

According to Amis (22), the most sensitive test for determining whether ion-dipole reaction rates are influenced virtually exclusively by electrostatic effects lies in their obedience to the expression

$$\Delta E_c = \frac{69.1\ zu}{D_1 D_2 r^2}\ \Delta D$$

where E_c is the "coulombic activation energy," D_1 and D_2 are the dielectric constants of the media; z, u, and r have the same significance given previously. Our inability to evaluate the parameters r and u accurately prevents any precise testing of our data.

The direct relationship between activation energy and dielectric constant in systems containing from 10 to 50% glycol is in accord with apparent theoretical requirements. Amis and Holmes (23) state that for a reaction between a dipolar molecule and a cation, a decrease in dielectric constant should be accompanied by a decrease in activation energy. The value of 19.5 Kcal./mole for the reaction in purely aqueous systems is, of course, quite out of line with the rest of the values. Since there are a number (23–26) of recorded instances in which the direct relationship for reactions between cations and dipoles did not hold, it is possible that a direct relationship is not a rigid requirement of theory.

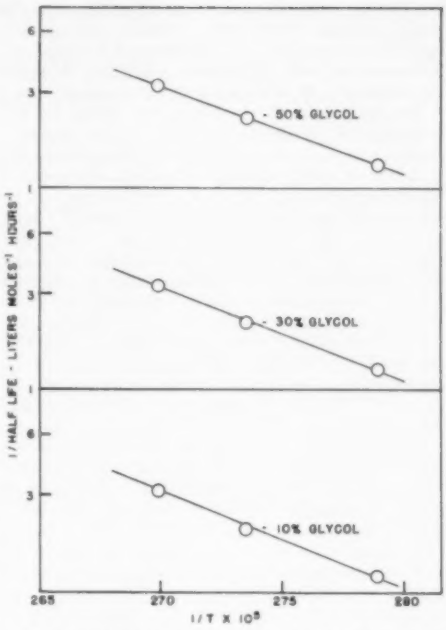


Fig. 8.—Arrhenius-type plots showing the temperature dependencies of the reactions in 10, 30, and 50% propylene glycol. The activation energies as determined from the slopes of these plots are: 21.0, 20.5, and 20.0 Kcal./mole for 10, 30, and 50% glycol, respectively.

We are not, therefore, inclined to attach any great significance to the direction of change in activation energy with decreasing dielectric constant which we have found. It would, perhaps, be more logical to point out that our values for the activation energies are subject to an error of ±1 Kcal./mole. As a result, the differences noted above may be of doubtful significance.

PHARMACEUTICAL SIGNIFICANCE

The preceding data, figures, and discussion make it clear that it is unwise to make any blanket assumption that replacement of all or a portion of the water in a pharmaceutical preparation will enhance the stability of an active ingredient. It is equally clear that the use of nonaqueous solvents to achieve greater solubility introduces the possibility of decreasing the stability of a potentially active ingredient. In order to evaluate any contributions which might be

due to a nonaqueous solvent, prior kinetic studies in purely aqueous and in mixed solvent solutions are virtually mandatory. A knowledge of both the *apparent* and *operative* mechanisms is similarly desirable if stability is to be predicted with any accuracy.

The present study serves well to point up the necessity for proper interpretation of any stability studies. It might be assumed, for example, that the inclusion of chloramphenicol in acidic solutions, of which propylene glycol is a constituent, could lead only to decreased stability of the antibiotic. Such apprehension might be particularly severe if a comparison were made of the relative stabilities in 0 and 10% propylene glycol, omitting studies in solutions containing higher concentrations of the glycol. Since the function of propylene glycol in such a preparation would undoubtedly be to increase the solubility of the antibiotic and thus permit therapeutically effective doses, a potentially useful solvent might be discarded because of insufficient information and misinterpretation of the available data.

For one thing, the inclusion of 10% propylene glycol will not increase the rate of degradation of chloramphenicol by more than 10%. This is not, of course, an insignificant factor and, from this superficial viewpoint, one might understandably be loathe to investigate systems containing higher concentrations of propylene glycol. This reticence might well apply even when it became clear that only by increasing the glycol concentration to 50% could therapeutically effective concentrations be kept in solution. Had the region between 10 and 50% glycol been investigated, however, the relative independence of the rate of degradation on glycol concentration would have been established. At that point, perhaps, a decrease in the stability of the antibiotic by 10% might have represented a small price to pay for achieving solutions containing therapeutically effective amounts in reasonable dose volumes.

The value of studies such as the present one may be seen from still another angle. A formulator aware of the mechanism proposed for the hydrogen ion catalyzed degradation of chloramphenicol in purely aqueous systems might, conceivably, attempt to enhance the stability of the antibiotic in solution by effecting a decrease in dielectric constant. Such an effort would be quite logical according to the Bronsted-Christiansen-Scatchard expression. Had he, however, used propylene glycol to achieve this effect, the results of the present study clearly show that his efforts would have been in vain. It is obviously important to be aware of the mechanism which is operative as well as the one which is apparent.

We do not wish to suggest that a solution of chloramphenicol in perchloric acid, no matter how dilute the acid, represents a plausible dosage form. We do wish to point out, however, that the factors important here may be of equal importance in systems more plausible as dosage forms containing active ingredients less resistant to degradation.

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A Comparison of the Kinetics of the Acid Catalyzed Hydrolyses of Procainamide, Procaine, and Benzocaine*

By ARNOLD D. MARCUS† and STEPHEN BARON

The kinetics of the acid catalyzed hydrolyses of procaine and benzocaine have been found to be first order with respect to the esters and the rates linearly dependent upon acid concentration. The catalytic constants for the acid catalyzed hydrolysis of procaine have been evaluated and compared with those obtained for procainamide. The differences in magnitudes have been compared and an attempt is made to explain these differences. The energies of activation have been determined to be 16.8 and 18.6 kilocalories/mole for procaine and benzocaine, respectively. The fact that the activation energy for procaine is 3.8 kilocalories/mole higher than for procainamide disproves a previous postulation that, in acid systems, procainamide responds as an ester rather than as an amide. The kinetics and mechanisms of the acid catalyzed hydrolyses of procainamide, procaine, and benzocaine have been compared and analyzed from a standpoint of the activation energies, frequency factors, and entropies of activation. The apparently "unreasonable" values of the frequency factors and activation energies of procainamide and procaine have been examined in terms of both collision and transition state theories. It appears that when the reactions are regarded to involve ions of like sign, the relative and absolute magnitudes of the frequency factors and activation energies are not "unreasonable."

IN A PREVIOUS COMMUNICATION (1) it was reported that, contrary to apparent theoretical requirements, the activation energy for the acid catalyzed hydrolysis of procainamide is a surprisingly low 13.0 Kcal./mole. In an attempt to explain this apparent deviation from theory within the framework of apparently well-established mechanisms (2, 3), it was proposed that, in acid systems, procainamide responds as an ester rather than as an amide. Since no experimental evidence concerning the validity of this postulation was available, it was noted that similar studies on procaine (the ester) and benzocaine would be initiated.

The present communication reports the results of a study of the kinetics of the acid catalyzed hydrolyses of procaine and benzocaine. If it could be shown that the activation energy for procaine is 13.0 Kcal./mole or lower, the postulation that procainamide responds as an ester would receive considerable support. On the other hand, if it were found that the activation energy for the acid catalyzed hydrolysis of procaine exceeds 13.0 Kcal./mole, a revision in the proposed mechanism would be necessary. Although it was felt that a study of benzocaine would be of some significance in proving or disproving the postulation, its major function seemed to be in permitting some evaluation of the contributions of the β-diethylammonium function.

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The authors wish to express their appreciation to Mr. Marvin Stern and Mr. Anthony Taraszka for their assistance with the calculations and for their many helpful discussions relative to the theoretical aspects of this study. They also wish to thank Dr. Gilbert S. Panson of the Department of Chemistry of Rutgers University for his aid in the theoretical aspects of this investigation.

EXPERIMENTAL

Procaine.—The general procedure and analytical method of Higuchi, *et al.* (4), were used throughout, with the following modifications: The withdrawn samples were diluted only tenfold at first with cold water. Aliquots of these dilutions were then diluted tenfold with 0.5 *M*, pH 9.5 carbonate-bicarbonate buffer just prior to spectrophotometric analysis. The degradations were effected in 0.25 to 1.0*M* perchloric acid solutions.

Benzocaine.—The same general experimental and analytical methods used for procaine were employed for benzocaine with the following modifications: Since benzocaine is poorly soluble in water, the 1% solutions of the ester were prepared with 0.5 *M* hydrochloric acid as the solvent. Because the specific extinction coefficient and wavelength of maximum absorption of benzocaine differ from those of procaine, 5.0 ml., rather than 10.0 ml., portions of 1% solution were added to the reaction flask. As a result of these modifications, the equation used to calculate the degree of hydrolysis must be rewritten as

$$\% \text{ residual ester} = 100 \times \frac{(k - k_{\text{PABA}})}{(k_{\text{benz.}} - k_{\text{PABA}})}$$

where

k = the observed absorbance of the partly hydrolyzed sample diluted to 0.000500% with respect to original benzocaine, at 285 mμ.

k_{PABA} = the absorbance of *p*-aminobenzoate ion equivalent to 0.00500% of benzocaine, at 285 mμ.

$k_{\text{benz.}}$ = the absorbance of a 0.000500% solution of benzocaine at 285 mμ.

All measurements were made at pH 9.5.

EXPERIMENTAL RESULTS

Figures 1 and 2 show the acid catalyzed hydrolyses of procaine and benzocaine to be first order with respect to the substrates over a range of acid concentration and temperature. The expected linear relationship between rate of hydrolysis of procaine and acid concentration is evident from Fig. 3. The catalytic constants for the hydrogen ion have been evaluated from the slopes of these plots and are included in Table I. The previously determined (1) constants for procainamide are also included for purposes of comparison.

Figure 4 shows that the rate of hydrolysis of benzocaine at 97.30° is linear with acid concentration. Although we have not investigated the relationship at lower temperatures, it is reasonable to assume similar dependencies on the basis of other studies (5, 6).

Temperature Dependencies.—The activation energies for the two acid catalyzed reactions have been determined from the slopes of the Arrhenius-type plots in Figs. 5 and 6. These are 16.8 and 18.6 Kcal./mole for procaine and benzocaine, respectively. Table II includes the first order rate constants, k; activation energies, ΔH_a; frequency factors, A; and entropies of activation, ΔS_a, for the acid catalyzed hydrolyses of procaine, procainamide, and benzocaine. The values for the latter two characteristics were calculated from the equation

$$k = \frac{RT}{Nh} e^{\Delta S a/R} e^{-\Delta H a/RT}$$

where

$$A = \frac{RT}{Nh} e^{\Delta S a/R}$$

For the sake of uniformity and ease of comparison the data in Table II all refer to reactions in 0.5 M perchloric acid at 97.30° and k has the dimension seconds^{-1}.

TABLE I.—CATALYTIC CONSTANTS FOR THE HYDROGEN-ION CATALYZED HYDROLYSES OF PROCAINE AND PROCAINAMIDE

T — °C.	k_H^+ Liters Moles^{-1} Hours^{-1}— Procaine	k_H^+ Liters Moles^{-1} Hours^{-1}— Procainamide
97.30	0.2775	0.0523
91.30	0.1975	0.0379
85.30	0.1265	0.0272

[a] Data of Marcus and Taraszka (1).

DISCUSSION OF THE EXPERIMENTAL RESULTS

Since the activation energy for the acid catalyzed hydrolysis of procaine is 3.8 Kcal./mole greater than for procainamide, we must conclude that the previously postulated mechanism for procainamide (1) is erroneous. A reconsideration of this mechanism will be presented in a subsequent section.

Benzocaine.—The value of 18.6 Kcal./mole for benzocaine is in general agreement with theoretical requirements in terms of substituent effects (5). The magnitude of the difference, however, seems small when regarded solely in terms of the electrophilic character of the *p*-ammonium substituent. In 50% ethanol the activation energy for the acid catalyzed hydrolysis of ethyl *p*-nitrobenzoate is, for example, 17.52 Kcal./mole (5). Since a *p*-ammonium function is more electrophilic than a *p*-nitro substituent, the value of 18.6 Kcal./mole for benzocaine must represent, in part, other than electrophilic effects. It would appear logical to assume that this value represents the net effect of electrophilic character and coulombic repulsion. The difference in solvent systems does, of course, play some part but the comparisons and conclusions remain valid.

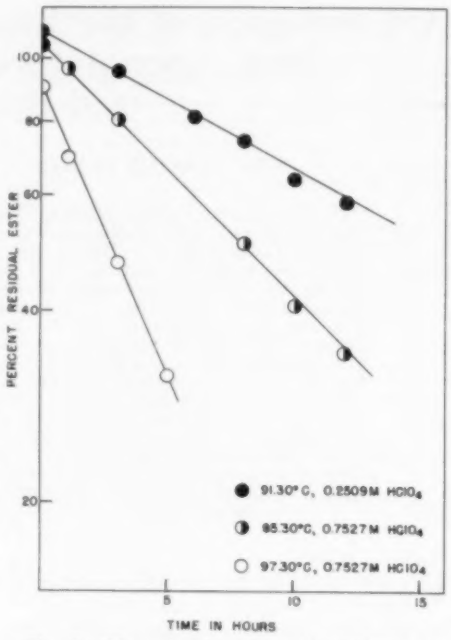

Fig. 1.—Plots showing the acid catalyzed hydrolysis of procaine to be first order with respect to the ester under a variety of experimental conditions.

TABLE II.—FIRST ORDER RATE CONSTANTS, ENERGIES OF ACTIVATION, FREQUENCY FACTORS, AND ENTROPIES OF ACTIVATION[a]

	k-sec.$^{-1}$	ΔH_a Kcal./ mole	A-sec.$^{-1}$	ΔS_a Entropy Units
Procainamide[b]	7.26	13.0	3.43×10^9	−47.4
Procaine	38.5	16.0	3.14×10^8	−33.8
Benzocaine	140.0	18.6	1.32×10^7	−26.4

[a] All values refer to 0.5 M perchloric acid at 97.30°.
[b] Calculated from the data of Marcus and Taraszka (1).

Procaine Versus Benzocaine.—The preceding discussions and comparisons indicate that coulombic repulsion may be important in determining the activation energy of a reaction. It is, therefore, difficult to reconcile the activation energies for benzocaine and procaine with the expected coulombic repulsions. In the perchloric acid solutions employed in the present study, procaine is present as a doubly protonated species whereas benzocaine is only monoprotonated. Since acid catalyzed hydrolysis requires an additional protonation (2, 3), it seemed reasonable to ex-

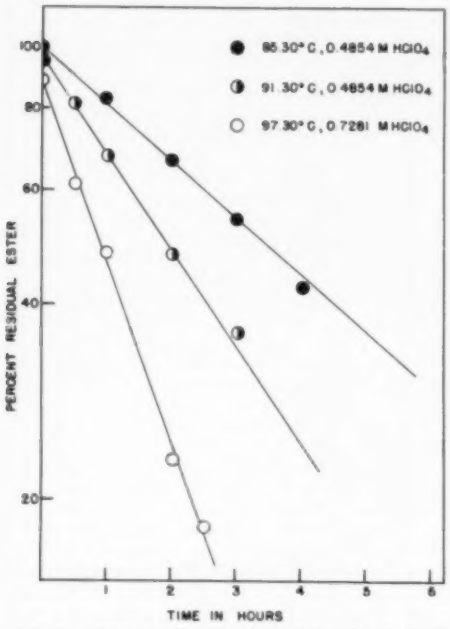

Fig. 2.—Plots showing the acid catalyzed hydrolysis of benzocaine to be first order with respect to benzocaine over a range of experimental conditions.

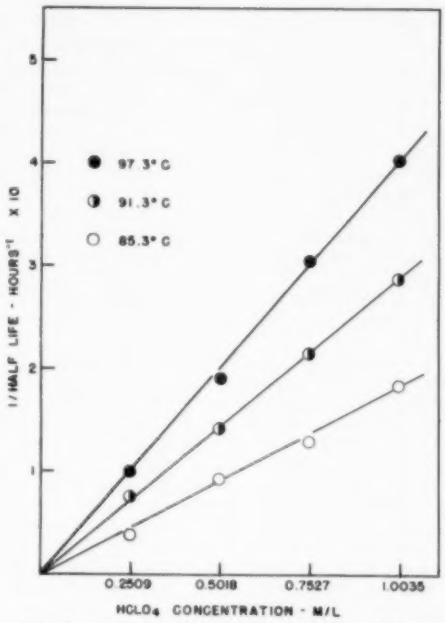

Fig. 3.—Plots showing the rate of the acid catalyzed hydrolysis of procaine to be linearly dependent upon the concentration of acid.

pect that the doubly protonated substrate would repel the catalytic species to the greater degree. The logical assumption which follows from this expectation would be that procaine should be associated with a higher activation energy than benzocaine.

Contrary to this assumption, the activation energy for the acid catalyzed hydrolysis is 1.8 Kcal./mole lower than for benzocaine. It is interesting to note that this is quite analogous to the acid catalyzed hydrolysis of procainamide in which every factor appeared to require an activation energy of perhaps 30 Kcal./mole. The experimentally determined activation energy is 13.0 Kcal./mole (1). Factors other than electrophilic substituents on the aromatic nuclei and coulombic repulsions are operative in these reactions. Indeed, it would seem that these additional factors are not only operative but that theirs are the predominating influences.

MECHANISMS OF THE REACTIONS

Influence on the Magnitudes of the Catalytic Constants.—Of all the data in Tables I and II, the differences in the magnitudes of the catalytic constants appear most amenable to treatment from straightforward considerations.

Since the present study apparently disproves the hypothesis that procainamide responds as an ester in acid systems, it must be assumed that the mechanism for the acid catalyzed hydrolysis of procainamide is of the usual type for benzamides. According to these apparently well established mechanisms (2, 3), acid catalyzed hydrolysis of amides proceeds through protonation of the amide nitrogen. It has been pointed out previously (1) that the β-diethyl-

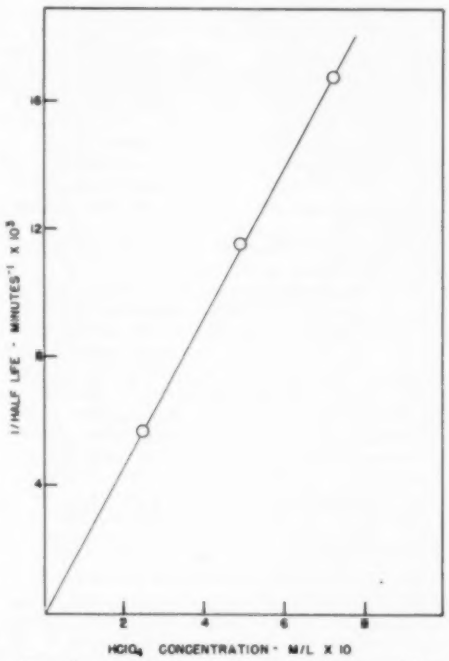

Fig. 4.—A plot establishing the direct relationship between the rate of the acid catalyzed hydrolysis of benzocaine and the concentration of acid at 97.30°.

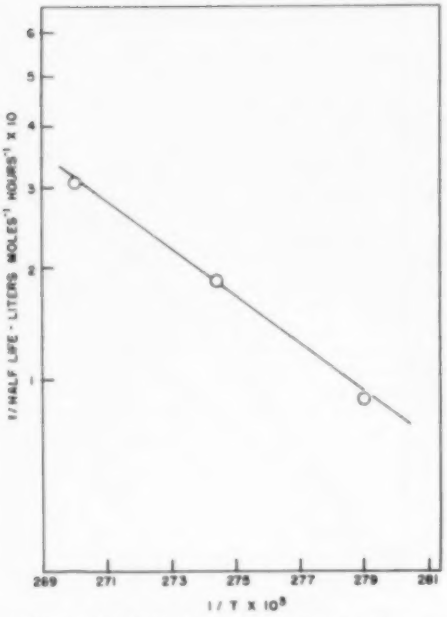

Fig. 5.—An Arrhenius-type plot showing the temperature dependency of the acid catalyzed hydrolysis of procaine. The activation energy for the reaction, as determined from the slope of this plot, is 16.8 Kcal./mole.

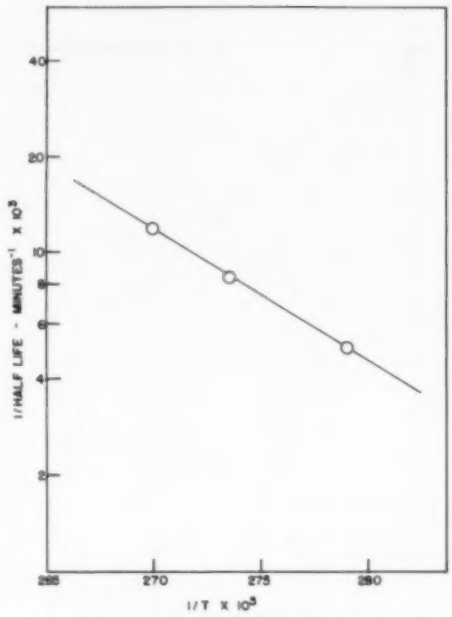

Fig. 6.—An Arrhenius-type plot showing the effect of temperature on the rate of the acid catalyzed hydrolysis of benzocaine. The activation energy as determined from the slope of the line is 18.6 Kcal./mole.

ammonium function is sufficiently electrophilic to markedly reduce the basicity of the amide nitrogen. As a result, protonation is more difficult and the efficacy of the catalytic species (H_3O^+) is decreased. This decrease is reflected in the magnitude of $k_H{}^+$. Since the acid catalyzed hydrolysis of esters also requires protonation (at the ester oxygen) we might expect $k_H{}^+$ to be almost the same for procaine and procainamide.

In the acid catalyzed hydrolysis of esters, however, even though protonation is important, it is much less important than in the hydrolysis of amides (2). On the other hand, in the acid catalyzed hydrolysis of an ester, attack by a water molecule at the carbonyl carbon is of much greater consequence than in amide hydrolysis (2). Inasmuch as the carbonyl carbon atom is much farther from the electrophilic β-diethylammonium substituent, the effect is materially reduced. In fact, the electrophilic character of the *p-ammonium function* would actually facilitate the acid catalyzed hydrolysis of an ester. Lastly, the effect of coulombic repulsion of the proton would be greater in the case of an amide than in the case of an ester. This results from the fact that protonation of the amide nitrogen, a site close to the formal positive charge, plays a much greater role in the hydrolysis of amides than in the hydrolysis of esters. There thus appear to be three different but related factors which account for the relative magnitudes of the catalytic constants.

Any rationalization or explanation of the values for the activation energies, frequency factors, or entropies of activation (Table II) presents a more difficult problem. When compared with the corresponding values for the acid catalyzed hydrolyses of ethyl *p*-nitrobenzoate or *p*-nitrobenzamide (2, 5) the frequency factor for procaine appears low by a factor of 10^3, and that of procainamide low by a factor of 10^8 or 10^9. Only benzocaine is associated with a frequency factor of "reasonable" magnitude. The large negative entropies of activation fall into a similar pattern and the activation energies are almost incredible. It is possible, however, that when viewed in a different light, the values for these reactions may appear more credible and reasonable.

Actual Nature of the Reactions.—By referring to a reaction between a proton and procainamide, procaine, or benzocaine, we leave the impression that the reaction involves an ion and a *dipolar molecule*. This is, of course, a false impression. The reactions are actually between ions of like sign. Since it is much easier to write "procaine" than β-diethylammonium-ethyl *p*-ammoniumbenzoate, we shall retain the former designation. We wish to emphasize, however, that the substrate is a diprotonated species. The same cautions hold for procainamide and benzocaine (mono-protonated). Since the reactions are between ions of like sign, the values for the frequency factors and the entropies of activation may not be "out of line" and may even help explain the variations in the activation energies.

Procainamide

The frequency factor, A, may be regarded as approximately equivalent to the pZ term of the collision theory, and the steric factor, p, is associated

with the entropy term. It is, therefore, possible to consider the unusually low values of A and activation energy for procainamide from two viewpoints.

Collision Theory.—If a collision between two reactants of proper energy content is to result in a reaction, the colliding species must be properly oriented with respect to each other. Unless they are so oriented, the energy content of the reactants may be of little consequence. It has been found that in reactions between ions of like sign, p may have a value as low as 10^{-9} (7). In the case of procainamide, the two protons already present could be expected to exert a considerable repulsive effect when a third proton approaches. This effect would result in sufficient steric hindrance to almost demand a very low value for p and, therefore, of pZ or A.

When this effect is considered together with the reduced basicity of the amide nitrogen, it is not at all difficult to imagine that the third protonation will require the strictest orientation of the reactants. Furthermore, even though attack by a water molecule at the carbonyl carbon is not so important as protonation of the amide nitrogen, this attack must also occur. As a result, the reaction requires orientation, not only with respect to the ions, but with respect to a molecule of water as well. It would appear, therefore, that the rate of acid catalyzed hydrolysis of procainamide is considerably more dependent upon proper orientation of the reactants than upon their energy content at the time of collision.

The orientation requirements are so strict that only a few collisions can be effective. When, however, the third protonation does take place, the product is a triply charged ion. Not only is the new entity triply charged, but the charges are so close together that the new species will, figuratively speaking, tend to tear itself apart despite a low energy content.

Transition State Theory.—A similar conclusion may be reached if the reaction is considered in terms of a transition state or activated complex. When ions of the same sign react, the product is a more highly charged ion. Solvation of the activated complex would then require many more solvent molecules than the individual reactants. The consequent reduction in the freedom of the solvent would lead to a substantial decrease in entropy (a large negative entropy of activation) in forming the transition state. This would, in turn, require a low value for the frequency factor and, as above, the frequency factor rather than the energy content of the reactants would predominate in determining the rate of reaction.

If the above reasoning is correct, the low values for A and ΔH_a for the acid catalyzed hydrolysis of procainamide are not particularly "unreasonable" but might actually be expected. In this connection, it is interesting to note the work of Jellinek and Gordon who have shown the actual substrate in the acid catalyzed hydrolysis of nicotinamide to be a doubly charged cation (8). The frequency factors given by these authors at 10^4 or 10^5 lower than for the acid catalyzed hydrolysis of *p*-nitrobenzamide (2). Here, too, the importance of the entropy term appears to be considerable. It must be pointed out, however, that the activation energy for the acid catalyzed hydrolysis of nicotinamide is approximately 7 Kcal./mole greater than for procainamide.

Procaine

From the preceding discussion it would seem logical to expect that the same reasoning should hold true for procaine. In part, it does. The value of A for procaine appears, as noted previously, to be low by a factor of 10^3. It is, therefore, reasonable to assume that the entropy term (or steric factor) remains very important in determining the rate of acid catalyzed hydrolysis. Two aspects of this comparison remain, however, to be explained. First, the activation energy for procaine is 3.8 Kcal./mole greater than for procainamide and, secondly, the frequency factor is higher by a factor of 10^3. It would thus appear that proper orientation of the reactants or a large negative entropy of activation can supply only part of the answer.

The answer may lie in the essential difference between the acid catalyzed hydrolyses of benzoates and benzamides. Since the electrophilic β-diethylammonium substituent can be expected to produce the same *relative* decrease in the basicities of the amide nitrogen and the ester oxygen, it is possible that protonation of the ester oxygen (the weaker base) will require greater energy on the part of the reactants. This would then be reflected in the magnitude of the activation energy. Furthermore, the p-ammonium substituent on the aromatic nucleus of the acid moiety can be expected to facilitate attack by a water molecule at the carbonyl carbon atom (2). This ease of attack may then be reflected, not in the activation energy, but in a relaxation of the strictness of the orientation requirements. As a result, effective collisions could occur more frequently and this would, in turn, lead to a higher value for A (pZ). Inasmuch as attack at the carbonyl carbon is of greater importance in ester hydrolysis, the above explanation appears to offer a reasonable means of rationalizing the differences between procaine and procainamide.

Benzocaine

The comparatively "normal" value for A in the acid catalyzed hydrolysis of benzocaine indicates that the entropy term is not the major influence in determining the rate of hydrolysis. It would appear that the absence of the β-diethylammonium substituent results in a concomitant absence of any great steric hindrance. The difference in the entropies of activation for procaine and benzocaine is certainly evidence in this direction.

Except for the apparent coulombic repulsion which decreases the effect of the electrophilic p-ammonium substituent, the mechanism for the acid catalyzed hydrolysis of benzocaine is comparatively uncomplicated.

SUMMARY AND CONCLUSIONS

1. The rates of the acid catalyzed hydrolyses of procaine and benzocaine are first order with respect to the esters. There is a linear relationship between the rates of reaction and the concentration of acid.

2. The activation energies for these acid catalyzed hydrolyses are 16.8 and 18.6 Kcal./mole for procaine and benzocaine, respectively. The mag-

nitude of these activation energies indicates that a previously proposed mechanism for the acid catalyzed hydrolysis of procainamide was in error and this mechanism has been reconsidered.

3. Although the frequency factors and the entropies of activation for procaine and procainamide appear to be low by factors of 10^3 and 10^9, respectively, the values obtained appear more reasonable when examined in light of both the collision and transition state theories and their requirements for reactions between ions of like sign.

4. It is shown that an apparent deviation from theoretical requirements for a high activation energy may be explained in terms of a dominating influence of the entropy change or the "steric factor" upon the rate of reaction.

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The Relative Reliability of the Escape Reaction and Righting-Reflex Sleeping Times in the Mouse*

By DUANE G. WENZEL and HARBANS LAL†

The "escape reaction" method of measuring the end point of sleeping time in mice was compared to the "righting-reflex" method. The former was found to be more reliable, to have a more definite end point, to measure more effectively lower levels of CNS depression, and to measure cortical rather than midbrain reflexes.

NUMEROUS METHODS have been used to quantitate drug-induced sleep. Probably the righting reflex is the most widely-used end point. Although the relative importance of, and the interrelationships between the many variables involved in sleep are still poorly understood, it is associated with both a decrease in the level of consciousness and an overall diminution in somatic motor activity (1). The important criteria for sleep have been stated to be: loss of critical, as opposed to stereotyped reactivity to the environment; an increased threshold of general sensibility and reflex irritability; and the capacity to be aroused or brought back to the state of consciousness (2–4). The recovery of postural reflexes and diminution of the threshold of general sensibility and reflex irritability appear to be brought about through the mesodiencephalic system (4). This system operates in the absence or incapacitation of the cerebral cortex employing feedback circuits to the lower centers and the periphery. Such reactions have been described in the decorticated cat, dog (4), and monkey (5), and new-born infants or older anencephalic children (4). Thus, although the righting reflex is a possible end point of sleep, it gives no evidence of cortical involvement.

In one attempt to improve the end point of sleeping time a mouse is placed in a cell the dimensions of which are so slected that only a fully-conscious animal can climb out of the cell (6). Stimuli are provided by intermittent blasts of air. This approach appears to bracket the target of pharmacological sleep as the animal must not only regain all reflexes and irritability in order to perceive the stimuli, but must consciously consider means of escape. Additionally, the act of climbing from the cell requires the recovery of neuromuscular coordination. The escape reaction should theoretically yield therefore a relatively complete evaluation of drug-induced sleep.

The primary purpose of this study was to compare the experimental accuracy of the escape reaction method to that of the righting reflex in the evaluation of drug-induced sleeping time. A related objective was to demonstrate the role of the cerebral cortex in both methods.

EXPERIMENTAL

Sleeping times of male mice (18–25 Gm.) were determined with the commonly used experimental barbiturates, hexobarbital and pentobarbital. Six mice were used for each dose, 10 doses being employed for hexobarbital and 11 for pentobarbital with each of the three tests. In all tests sleep was

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TABLE I.—ANALYSIS OF VARIANCE

Source of Variation	Righting Reflex		Escape Reaction	
	Degrees of Freedom	Mean Square A[a]	Mean Square B[b]	Degrees of Freedom	Mean Square
Hexobarbital Sodium					
Between doses	9	2.1353	2.0095	9	0.3529
Linear regression	1	14.8980	14.5610	1	2.8773
Deviation from linearity	8	0.5400	0.4406	8	0.0373
Error	49	0.1008	0.1239	50	0.0158
Total	58			59	
Pentobarbital Sodium					
Between doses	10	0.7512	0.6709	10	0.5533
Linear regression	1	6.8911	6.1422	1	5.1286
Deviation from linearity	9	0.9890	0.0630	9	0.0452
Error	55	0.0405	0.0428	53	0.0206
Total	65			63	

[a] First spontaneous righting. [b] Righting ability confirmed by repeated testing.

measured as the time interval between the intraperitoneal administration of the drug and the end point.

The escape reaction test was as previously described (6) except that six cells were employed simultaneously and an air reservoir of a heavy balloon was used. As some mice do not react to the air-blast stimulus, animals were first screened to eliminate those that did not come out of the cell within one hundred seconds. Two methods of recording the end point of the righting reflex were used. One employed the first spontaneous movement from the back to a normal position while in the second the animal was immediately returned to the back position. If the mouse remained in this position for at least thirty seconds, the awakening was regarded as a sleep movement and timing continued. The end point was two returns to the normal position each within the prescribed thirty-second interval.

Six mice which had demonstrated an escape reaction to the air blast were carefully decorticated and tested by both methods.

RESULTS AND DISCUSSION

The regression analyses of variance performed on the sleeping times are given in Table I from log-transformed data. It is seen that the two righting-reflex methods do not differ significantly. When the error of the initial spontaneous righting method, which is the smaller of the two, is compared to the error of the escape reaction it is found that the latter is significantly smaller. The level of significance is 0.1% in the case of hexobarbital and 1.0% for pentobarbital. When the data for each group of animals were individually examined, it was found that the lesser significance obtained with pentobarbital was the result of data from the higher doses employed. It would appear that the variance of the escape reaction method becomes greater with the increased duration of sleeping time. It was also observed that with the two smaller doses of hexobarbital (76 and 87 mg./Kg.) that only five out of twelve mice lost the righting reflex whereas the escape reaction test satisfactorily measured the shorter sleeping time.

The decorticated mice irreversibly lost the ability to perform the escape reaction but retained the righting reflex and other midbrain reflexes. This information, coupled with the longer sleeping times obtained with the escape reaction method, corroborates the initial assumption that this constitutes a more reliable criterion of cortical involvement than the righting reflex.

It may be concluded that the escape reaction test employed provides a more valid approximation of drug-induced sleeping time than the righting reflex method. Furthermore, it has an unmistakable end point, a smaller error, and effectively measures lower levels of CNS depression.

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Pharmaceutical Applications of the Sodium Salt of Carbopol 934*

By JAMES A. LEE and W. LEWIS NOBLES†

This study indicates that a number of medicinal agents can be satisfactorily suspended using the dry sodium salt of Carbopol 934. The emulsifying properties of this agent are also reported.

THIS STUDY was undertaken in an effort to evaluate the suspending and emulsifying properties of the dry sodium salt of Carbopol 934,[1] (hereinafter designated as Carbopol). Of particular interest was the comparison of the behavior of this form of the agent with such properties previously reported (1) for both the unneutralized polymer and for preparations in which the acidic polymer was neutralized *in situ*.

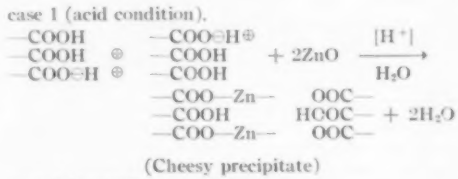
Carbopol is a hydrophilic colloid originally designed as a thickening agent for various types of aqueous formulations. It is a high molecular weight polymer containing a large percentage of carboxyl groups. Essentially, it is a carboxylic vinyl polymer supplied as a finely divided white powder. It disperses readily in water to yield an acid solution of low viscosity.

CARBOPOL AS A SUSPENDING AGENT

Previous publications (1, 2) have indicated methods by which the agent may be most easily dispersed and neutralized to serve as a suspending agent and emulsion stabilizer. That it can function effectively as both a primary emulsifier and emulsion stabilizer is a tribute to the unique gel-forming properties of this material. Once the desired agent has been dispersed, coalescence is effectively prevented by electrical charge repulsions within the molecule. The mechanisms responsible for the very high viscosities obtained are solvation and electrical charge repulsions within the molecule. Unfortunately, however, because of the latter effect, ionic materials added to a Carbopol-thickened system supply cations which are attracted to and surround the negative charges of the molecule. The rigid electrical repulsion within the molecule is thereby lessened and the result is a marked lowering of viscosity unless the concentration of Carbopol is increased to offset the loss of optimum viscosity produced by the cations (4).

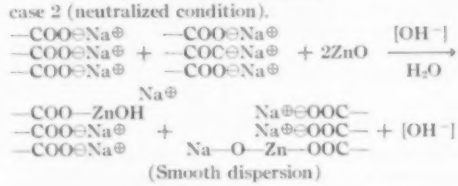
Carbopol salts are excellent suspending agents for many solids dispersed in aqueous systems. Satisfactory dispersions of calamine, neocalamine, and a kaolin-pectin combination were previously reported. Other workers (1, 3), however, had reported that certain multivalent metallic ions were poorly suspended. It had been reported (3) that cosmetic and pharmaceutical applications of this agent had been hindered by the reaction of the multivalent metallic ions to form insoluble salts of Carbopol, resulting in unsightly, coarse preparations. Some of these findings were in apparent conflict with our earlier observations relative to calamine, neocalamine, and zinc oxide (2). Nevertheless, on reexamination, our earlier findings were confirmed. During the past year, Cohen (3) presented a theory of salt formation which may serve satisfactorily as a basis for the interpretation of the results obtained with such substances. The following equations thus are suggested as possible explanations of some of the difficulties previously encountered in the use of multivalent metallic ions with Carbopol:

case 1 (acid condition).



(Cheesy precipitate)

case 2 (neutralized condition).



(Smooth dispersion)

While it is readily admitted that these equations are hypothetical in nature, they appear to interpret, fairly accurately, the results obtained with the use of Carbopol in such formulations. In case 1, the formation of a cheesy precipitate with no viscosity in the liquid phase tends to support the concept of the formation of —COO—Zn—OOC groups upon the addition of zinc oxide to the acidic polymer. In case 2, there is no direct evidence to support the conjecture that the —COO—Zn—OH group is actually present. Brown and Duke (5), however, suggest the presence of such a linkage as being involved in the vulcanization of carboxylic rubber with zinc oxide. Furthermore, this type of grouping is analogous to basic zinc acetate, $CH_3COO—Zn—OH$. In the presence of the sodium ion there is also the possibility of —COO—Zn—ONa groups being present.

When the metallic oxide is added to a non-neutralized dispersion of Carbopol, an unsatisfactory dispersion of agglomerated particles may result, as suggested in case 1. If, however, the

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[1] A product of B. F. Goodrich Chemical Co.

Carbopol is neutralized to a pH of 7 or higher, prior to the addition of such agents, a smooth, stable dispersion may be obtained.

The following data (3) suggest that the poor suspending property of Carbopol in the presence of zinc oxide is due to the neutralization of this agent by the zinc oxide: (*a*) The pH of a 1% dispersion of zinc oxide in water is 6.9. (Taken alone this would not predict a basic reaction.) (*b*) The pH of a 1% dispersion of Carbopol 934 in water is 3.1, but (*c*) when 1.65 Gm. zinc oxide is added to 300 Gm. of a 1.0% Carbopol dispersion (equimolar amount of zinc oxide and Carbopol) and mixed in a Waring Blendor for thirty minutes, a grainy precipitate results, and the pH is raised to 6.3. This is positive proof of the zinc oxide neutralization of the carboxylic acid groups on the Carbopol and, in conjunction with the formation of a precipitate, strongly indicated the equation in case 1. (*d*) If sodium hydroxide solution is added to the precipitated formulation resulting in (*c*), and mixed for a very long time, the precipitate will disappear and the viscosity of the liquid phase will eventually increase greatly, indicating the disappearance of the —Zn— crosslinks in a manner as postulated in case 2. The following data demonstrate this effect:

10% NaOH Added to 300 Gm. of Resulting Formulation in (*c*), ml.	Total Time Mixed in Waring Blendor at Full Speed, min.	pH	Brookfield 20 r. p. m. Viscosity	Comments
5.0	44	9.8	0	Ppt. much finer
13.0	116	11.5	0	Ppt. much finer
13.0	127	10.8	19,120	Smooth liquid phase, thick by paper test, no precipitation or agglomeration
	Sample aged 48 hours, min.			
15.0	127	9.8	17,800	Smooth, as above
15.0	134	11.4	8,000	Smooth, as above
10% acetic acid added	...	6.2	0	Grainy ppt. reforms, no viscosity

Thus, a good suspension can be formed by adding zinc oxide to acidic Carbopol followed by sodium hydroxide addition, but a tremendous amount of mixing must be used to eliminate the —COO—Zn—OOC— groups, once they are formed. The degree of mixing required is completely impractical and would appear to be of academic interest only.

(*e*) On the other hand, the use of preneutralized Carbopol mucilage eliminates the formation of precipitate (—COO—Zn—OOC—) as occurred in (*c*) and avoids the necessity of the tremendous amount of mixing employed in (*d*)

This may be illustrated by the following results utilizing the same ingredients:

	pH	Viscosity, c.p.s.
300 Gm. of a 1% Carbopol dispersion plus 10 ml. 10% NaOH	7.1	42,500
1.65 Gm. of zinc oxide added with three minutes mixing on Waring Blendor	8.5	34,200 (smooth, good)
10% additional zinc oxide added to formulation	8.6	36,500 (smooth, good)
Aged twenty-four hours	8.6	46,000 (smooth, good)

In the course of the present preparative work, the dry sodium salt of Carbopol was utilized. This was prepared as reported elsewhere (6). By this method approximately 0.4 Gm. sodium hydroxide is used per gram of Carbopol. This ratio of alkali/Carbopol will produce a mucilage possessing a pH range of 7–7.6.

The results of this study are summarized in Table I. They may be compared with the results reported by Skauen and his co-workers (1).

The dry sodium salt of Carbopol works well in this type of application. This agent produces a satisfactory suspension and obviates the necessity of using any previously prepared solutions or the weighing of small quantities of the neutralization agent each time. The substances to be suspended can be dry blended (in the present work this was done on small runs in a mortar and pestle and on larger runs with the use of the Patterson-Kelley yoke type blender) with the sodium salt and then water may be added with trituration to give the desired volume.

The second phase of this study dealt with the use of Carbopol as a primary emulsifier and/or an emulsion stabilizer. This agent has been recommended and has found commercial application as an emulsion stabilizer. Skauen and his associates (1) reported its use as an emulsifying agent when used with such substances as liquid petrolatum, cod liver oil, cotton seed oil, and turpentine oil.

When this agent is used as an emulsion stabilizer in a system involving other emulsifying agents, excessive amounts of Carbopol are necessary due to the ionic deswelling produced by the sodium ion, if the other agent be sodium stearate, sodium oleate, or a similar substance. Such emulsions are easily prepared by either the "dry" or "wet" gum method. To prepare an emulsion by the dry gum method, the oil was placed in a dry mortar and the desired quantity of the sodium salts was thoroughly incorporated. Water was then added, in portions; emulsification taking place while triturating. The appearance of the preparation improved considerably after it had been passed through a hand homogenizer several times.

To utilize the wet gum method, a mucilage was prepared from the dry sodium salt by the addition of water with constant agitation. The oil was added, in portions, with constant trituration. In this case also, the use of the hand homogenizer greatly improved the appearance of the product.

These emulsions may be more conveniently pre-

TABLE I.—SUSPENSIONS PREPARED USING DRY SODIUM SALT OF CARBOPOL 934 (OBSERVATIONS MADE AFTER NINETY-SIX HOURS)

Substances Used	Carbopol Salt 0.25%	0.5%	0.75%
Oil of pine tar, 5%; triethanolamine, 1.5%	Slight separation Shakes in well	No separation	.....
Coal tar, 5%	5% separation, good	No separation, too thick	.....
Ichthammol, 10%; glycerin, 3%	No separation, good	.....	.....
Sulfur, 5%; glycerin, 3%	5% separation, good	No separation, good	.....
Starch, 10%; glycerin, 3%	40% separation, poor	No separation, good	.....
Benzocaine, 5%; neocalamine, 16%; glycerin, 3%	.....	No separation, good	.....
Bismuth subcarbonate, 5%; glycerin, 3%	Slight separation, good	No separation, good	.....
Zinc oxide, 10%; glycerin, 3%	.....	30% separation, poor	0.65% Carbopol salt, sligat separation, good
Aspirin, 8%	.....	Granular separation	Poor
Calamine, 8%; Zinc oxide, 8%; Glycerin, 4%	Granular, poor	0.65% Carbopol salt, no separation, good	.....

TABLE II.—EMULSIONS USING DRY SODIUM SALT OF CARBOPOL (OBSERVATIONS MADE AFTER ONE HUNDRED TWENTY HOURS)

	Percentages of Carbopol Salt Used 0.15	0.25	0.5	1	2
Liquid petrolatum, 50%	.....	No separation	.....	No separation	.....
Cod liver oil, 50%	2% separation	No separation	.....	.....	.....
Benzyl benzoate, 25%	.....	20% creaming	15% creaming	No separation	.....
Cottonseed oil, 50%	.....	35% separation	30% separation	No separation; too thick	.....
Turpentine oil, 15%	.....	.....	.....	No separation	No separation

pared in a mixer (such as a Waring Blendor) by adding the oil and mucilage followed by high speed agitation.

The results of our studies, utilizing the dry sodium salt, are presented in Table II.

The advantage and speed of the present method are emphasized as being more desirable than other methods (1) which require the use of a solution of the neutralizing agent of the desired percentage strength or the weighing, each time, of the proper quantity of neutralizer.

SUMMARY

Satisfactory suspensions and emulsions of a number of commonly employed medicinals were prepared with the use of the dry sodium salt of Carbopol. In general, 0.5% of the dry sodium salt appears to be the optimum concentration for suspending purposes.

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Sterility of Antibiotic Ophthalmic Ointment*

By FRANCES W. BOWMAN and SIMON HOLDOWSKY

A survey was conducted to determine the sterility of antibiotic ophthalmic ointments. Forty-six samples representing nineteen batches were tested. Only 10 per cent of the batches were found contaminated, in contrast to approximately six times that percentage reported by other investigators.

THIS STUDY was prompted by the recent findings of Vander Wyk and Granston (1) that of twenty-eight samples of antibiotic ophthalmic ointments tested, seventeen were nonsterile. While the antibiotic regulations[1] governing the certification of such preparations do not require sterility testing, the Food and Drug Administration does insist that the manufacturing facilities be capable of producing essentially sterile products. Under the certification program (2), antibiotic ointments were tested for microorganism count from 1947 to 1950. During that time it was found that the incidence of contamination in ointments was very low. This finding resulted in a recommendation for discontinuance of such tests, and they were deleted from the antibiotic regulations in 1950 (3).

The method employed by Vander Wyk and Granston (1) differed from the former official method (2), and appeared to offer certain advantages in the efficiency of organism recovery. The old method consisted simply of squeezing approximately 0.1 to 0.5 Gm. of ointment on to the surface of 20 ml. of nutrient agar in a Petri dish. After the ointment was spread evenly over the agar with a sterile glass rod, the Petri dish was incubated at 37° for forty-eight hours. From the number of colonies appearing on the plate, the number of viable microorganisms per gram of ointment was calculated. The method of Vander Wyk and Granston introduces a dispersion step in which the ointment is shaken for one hour at room temperature with sterile glass beads in 25 ml. of distilled water. Three 1.0-ml. aliquots are then placed in Petri dishes, mixed with melted blood agar, and incubated at 37° for twenty-four hours.

EXPERIMENTAL

Forty-six commercial tubes of antibiotic ophthalmic ointment were tested by the procedure of Vander Wyk and Granston (1). These represented 19 batches from 10 manufacturers. All tubes were labeled to contain 1/8 of an ounce of ointment.

Results.—As shown in Table I, contamination was detected in only two batches, both from the same manufacturer. In one of the batches, one tube tested was sterile while the other was not. In the other contaminated batch, six tubes were tested and all were found to be nonsterile. Both bacteria and molds were observed in each case. That these findings were not due to chance contamination is supported by negative findings with nine other batches, seven in duplicate, one in sextuplicate, and another with eight replicates.

TABLE I.—STERILITY TESTS ON ANTIBIOTIC OPHTHALMIC OINTMENT

Type of Ointment	Organisms/Gm.
Penicillin G, 100,000 units/Gm.	
Batch 1 (1 tube)	0
Batch 2 (1 tube)	0
Batch 3 (2 tubes)	0
Penicillin G, 10,000 units/Gm. (1 Batch, 2 tubes)	0
Penicillin G, 1,000 units/Gm.	
Batch 1 (2 tubes)	0
Batch 2 (2 tubes)	0
Bacitracin, 500 units/Gm.	
Batch 1 (6 tubes)	0
Batch 2 (8 tubes)	0
Chloramphenicol, 1%	
Batch 1 (1 tube)	0
Batch 2 (1 tube)	0
Chlortetracycline Hydrochloride, 1%	
Batch 1 (1 tube)	0
Bacitracin, 500 units/Gm., and Neomycin Sulfate, 5 mg./Gm.	
Batch 1 (2 tubes)	0
Bacitracin, 500 units/Gm., and Polymyxin B Sulfate, 10,000 units/Gm.	
Batch 1—tube 1	0
Batch 1—tube 2	79
Batch 2—tube 1	30
Batch 2—tube 2	84
Batch 2—tube 3	28
Batch 2—tube 4	22
Batch 2—tube 5	22
Batch 2—tube 6	42
Bacitracin, 400 units/Gm., Neomycin Sulfate, 5 mg./Gm., and Polymyxin B Sulfate, 5,000 units/Gm.	
Batch 1 (2 tubes)	0
Batch 2 (2 tubes)	0
Batch 3 (2 tubes)	0
Bacitracin, 500 units/Gm., Neomycin Sulfate, 5 mg./Gm., and Polymyxin B Sulfate, 5,000 units/Gm.	
Batch 1 (2 tubes)	0
Bacitracin, 500 units/Gm., Neomycin Sulfate, 3 mg./Gm., and Polymyxin B Sulfate, 10,000 units/Gm.	
Batch 1 (1 tube)	0

DISCUSSION

Our findings do not fully corroborate those of Vander Wyk and Granston. Assuming that each of

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[1] The antibiotic regulations are issued under section 507 of the Federal Food, Drug, and Cosmetic Act.

the 28 samples tested by them represented a different batch, only 39% of the antibiotic ophthalmic ointments were sterile. Of the 19 batches tested by us, about 90% were sterile, a finding which agrees essentially both with four years' experience in testing antibiotic ointments from 1947 to 1950 and with results of a survey of ointments containing sulfonamides (4).

SUMMARY

Forty-six samples of antibiotic ophthalmic ointments were tested for sterility. Of the 19 batches represented, 90 per cent were sterile. This would indicate that the status of these ointments respecting the degree and incidence of contamination is more satisfactory than would be gathered from the results obtained by Vander Wyk and Granston.

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Influence of Dissolution Rate and Surface on Tetracycline Absorption*

By EINO NELSON

With the Technical Assistance of YOKO YUZURIHA

Tetracycline absorption was studied by means of excretion rate measurements in humans after 200-mg. doses of tetracycline or its salts. A direct correlation was found between excretion rate at one, two, and three hours and *in vitro* solution rate of the drugs when the dose was given in the form of two 0.95-cm. diameter pellets. Differences in rate were less noticeable when the dose given consisted of particles having an average diameter of 100 microns. The absorption of tetracycline, tetracycline phenolsulfonphthaleinate, and tetracycline sodium hexametaphosphate complex was rate-limited by *in vivo* solution rate to some degree. Tetracycline hydrochloride absorption was rate-limited by the absorption process itself. The bases for these correlations were discussed. The mathematics of urinary excretion was considered to the extent of pointing out the relationship between excretion rate and blood level.

THE ABSORPTION of tetracycline after oral ingestion by humans has recently been the subject of many investigations (1–7). These studies have dealt, for the most part, with serum levels obtainable when tetracycline or its salts were taken with or without additives by the oral route. With the exception of the study in which rather marked depression in serum levels was obtained when tetracycline hydrochloride was given with dicalcium phosphate (4), no clear picture has emerged to explain what factor or factors control the absorption of tetracycline.

In all of the investigations cited above, no attempts were apparently made to control or characterize the physical form of tetracycline in the capsules used as the dosage form. On the basis of particle size alone, which determines initial surface presented to dissolution mediums after ingestion, differences in blood levels should reasonably be expected in accordance with the results found in studies on sulfonamides (8, 9). In these two studies, micronized powdered sulfonamides gave higher levels than microcrystals. Another consideration in absorbability relates to the intrinsic solution rate properties of the same base compound in the form of different salts. This consideration, as well as the expected effect of other factors influencing solution rate of drugs at absorption sites, has been discussed recently (10–12).

It is only reasonable to expect that physical and physical-chemical factors should influence tetracycline absorption when the nature of the absorption process itself is considered. According to a recent report (13), tetracycline appears to be absorbed by free diffusion. An "absorption enhancement" due to additives or a particular salt used could not occur in this case since this would require involvement with a transport mechanism in absorption. Even if a transport mechanism were involved in tetracycline absorption, availability of drug in solution at absorption sites would still be required and it is conceivable that the rate-limiting step could be the rate of the solid to solution step. With absorption by free diffusion, the overall absorption process is even more susceptible to being controlled in rate by the rate of dissolution of drug at

* Received June 11, 1958, from the School of Pharmacy, University of California Medical Center, San Francisco 22.

absorption sites because of the concentration dependency of this process.

In comparative studies of the type which have been made on tetracycline, the considerations of effect of chemical nature of drug on absorbability are not pertinent since, regardless of the salt or mixture used, the same drug is involved in absorption. The dependency of absorption on pK and fat solubility of drugs is discussed (14–16) and the hypothesis advanced is involved in tetracycline absorption as such.

Another factor could have an influence on the overall absorption process of tetracycline. This is concerned with presence of materials in gastrointestinal tract capable of binding tetracycline and reducing its availability for absorption. Possibly, additives given with tetracycline could inhibit or prevent this occurrence, but no judgment of effectiveness is possible until the influence of the physical and chemical factors also involved is controlled and reproduced for successive trials.

The present report gives the results of excretion rate studies on tetracycline given in several different chemical and physical forms and discusses correlations that exist between these results and the physical-chemical property of solution rate and factors affecting this rate. This report also discusses the nature of the mathematical relationship between excretion rate and blood level.

EXPERIMENTAL

Materials and Assay.—The substances used in tests included tetracycline hydrochloride, tetracycline sodium hexametaphosphate complex, and tetracycline.[1] Some tests were also made using the phenolsulfonphthalein salt of tetracycline.[2] The activity of these preparations, when assayed by the cylinder-plate method (17) using *Bacillus cereus* var. *mycoides* as the test organism, is shown in Table I. This was also the assay procedure for urine samples. Urine samples were kept at 1° until diluted with 0.1 *M* monobasic potassium phosphate. Dilutions were usually made within twenty-four hours. Diluted samples were also stored at 1° and usually assayed within three days or less. Blank urine samples were treated and assayed in the same manner.

Subjects.—The test panel consisted of ten adult humans all in apparent normal health.[3] The ages and weights of the subjects are given in the first entry in Table II. The ten subjects were not all used in every cross comparison. Their participation in tests is indicated by the entries in Tables II and III.

Conduction of in Vivo Tests.—The dosage forms were taken on either a fasting stomach in the morning or at least one hour after a toast and coffee breakfast according to the individual preference of the subjects. No food was taken for at least one hour after ingestion of the dosage form. Each subject was instructed to drink water in an amount sufficient to allow him to collect urine samples at least at one, two, four, and eight hours. The volumes voided were noted and an aliquot taken for assay. One week usually elapsed between tests.

Preparation of Dosage Forms.—Two types of dosage forms were used. In the first type, two pellets of each material 0.95 cm. in diameter and about 0.15 cm. thick, pressed at about 1000 Kg./sq. cm. and containing 200 ± 10 mg. of tetracycline hydrochloride activity were packed in 000 capsules with 200 mg. sodium bicarbonate interspersed between the pellets and in the ends of the capsule. The pressings were made by a technique previously described (11). In the second type of dosage form, small particles made by grinding pellets of the type described above and sieving to collect the 115/200 (Tyler series) mesh fraction were packed in 00 capsules after loosely mixing with 200 mg. sodium bicarbonate. Each capsule contained 200 ± 1 mg. tetracycline hydrochloride equivalent. Prior to placing pellets in capsules or grinding to prepare small particles, the lubricant remaining on their outer surfaces as a result of the compression process was removed by rubbing with a fine grade emery cloth. The dosage forms were taken within a day of preparation to avoid reaction between the salts and sodium bicarbonate.

The pellets formed by the process described were quite hard. Soaking in water at room temperature did not cause excessive disintegration in times up to two hours.

In Vitro Solution Rate Tests.—The same materials used in *in vivo* studies were compressed into pellets 1.3 cm. in diameter and about 0.3 cm. thick by the technique and at the same pressure used in preparing the smaller pellets for *in vivo* tests. The dissolution rates of these pellets at 37 ± 2° in simulated gastric fluid (18) and in two simulated intestinal fluids (19) were measured by a free convection method (11). The simulated intestinal fluids were the neutral and alkaline solutions described in the cited report (19).

RESULTS AND DISCUSSION

Excretion Data.—Tables II and III summarize the excretion data collected in all tests including volumes of urine collected. Since it was not practical always to collect urine specimens at the even hour headings shown in these tables, the tabulated data represent values read at the stated intervals from individual plots of cumulative amounts excreted with time. The urine volume data for table entry were obtained in the same way except that linear interpolation was used to find volumes at even hours. With tetracycline excretion data, interpolations were made on the best line through

[1] These pure materials were supplied at various times by the Bristol Laboratories, E. R. Squibb and Sons, and the Chas. Pfizer Co. The author is grateful for this assistance. The tetracycline sodium hexametaphosphate complex was supplied by the Bristol Laboratories.

[2] Made by mixing aqueous solutions of tetracycline hydrochloride and the monosodium salt of phenolsulfonphthalein in stoichiometric quantities, collecting the precipitate, washing with several volumes of cold water, and drying over sulfuric acid in partial vacuum at room temperature. The dried material was used without further purification in all tests. This compound will be referred to as tetracycline PSP.

[3] This work was facilitated by a most cooperative test panel to whom the author is indebted.

the experimental points. This procedure was desirable since the amount excreted at various fixed times was necessary for interpretation of results.

Interpretation of Excretion Data.—The potential of excretion data to evaluate drug absorption does not appear to be generally recognized in spite of a considerable amount of data in the literature describing drug absorption, distribution, and excretion kinetics validating its use (see, for example, references 20–23). For those drugs that are eliminated in whole or in part unchanged in the urine and that show an exponential decay in concentration in the blood stream after drug in blood is in equilibrium with the other fluids of distribution, excretion rate is directly proportional to the amount of drug in the blood. Hence,

$$\frac{da_e}{dt} = ka_b \qquad \text{(Eq. 1)}$$

where a_e is the amount excreted in time t, a_b is the amount of drug in the blood, and k is a constant characteristic for a given drug and incorporates the specific elimination rate constant and the fraction of drug eliminated via the kidney (25). Equation 1 assumes that clearance does not change with urine elimination rate.

In some of the work described in this paper, it was not necessary to know the value of the constant k since it canceled in any comparison which was a ratio of excretion rates. Thus, when mean excretion rates at a given time from two preparations were compared, their ratio was the ratio of blood levels at the same time.

TABLE I.—ACTIVITY OF PREPARATIONS USED IN STUDIES[a]

Preparation	Theoretical	Found
Tetracycline HCl	100	100
Tetracycline sodium hexametaphosphate complex	...	76
Tetracycline PSP	60	63
Tetracycline	108	90

[a] Expressed in terms of per cent of activity of a sample of commercial tetracycline hydrochloride taken as standard.

TABLE II.—EXCRETION DATA FROM SINGLE 200-MG. DOSES OF TETRACYCLINE IN PELLET FORM[a]

Cumulative mg. of Tetracycline and ml. Urine Excreted to Various Times in Hours

Subject[b]	mg. as Hydrochloride 1.0	2.0	3.0	4.0	6.0	8.0	Urine Volume, ml. 1.0	2.0	3.0	4.0	6.0	8.0
Tetracycline PSP												
E (39–77)	1.0	5.6	10.0	13.1	18.1	22.0	740	1080	1240	1350	1470	1600
S (33–57)	0.5	3.8	8.6	14.0	19.3	21.5	60	120	190	270	430	520
B (28–82)	0.4	3.0	5.8	7.9	11.3	14.2	30	60	90	120	200	280
T (23–75)	1.0	4.4	8.6	11.7	16.5	20.1	60	110	160	200	220	340
N (21–77)	0	4.0	13.5	21.3	34.5	45.6	80	200	270	340	440	540
H (26–73)	0.5	2.5	5.9	9.8	14.7	17.8	50	170	450	740	830	930
L (30–59)	0.2	2.1	5.2	8.8	12.6	14.2	50	200	370	540	820	1070
A (24–84)	0.2	3.8	8.5	12.4	18.3	23.9	40	120	200	290	520	750
J (23–61)	1.1	4.2	6.2	7.6	9.0	9.5	150	460	560	660	770	870
R (26–84)	0.3	1.6	4.7	8.0	12.1	13.5	40	60	80	110	140	180
Mean	0.5	3.5	7.7	11.5	16.6	20.2	130	258	361	462	584	708
Tetracycline Sodium Hexametaphosphate Complex												
E	0.5	4.5	11.0	16.8	25.9	33.0	500	650	790	880	1020	1150
S	2.0	11.3	17.1	21.5	28.0	33.0	50	60	140	160	270	380
B	0.2	2.6	6.2	11.0	21.3	30.6	30	70	110	150	300	490
T	0.3	4.6	10.6	15.0	21.0	25.6	30	70	110	150	230	300
N	4.7	9.8	17.3	26.0	37.4	45.1	490	790	870	950	1090	1210
H	0	0.6	2.3	4.9	11.0	17.9	30	60	130	740	340	460
L	0.1	1.7	4.4	7.7	12.5	15.9	30	80	100	130	200	260
A	1.5	6.8	13.8	24.1	32.8	40.0	70	230	450	690	800	920
J	1.6	8.5	14.8	19.2	26.2	32.7	40	90	150	220	380	540
R	0.3	3.0	6.5	11.0	21.5	32.8	40	120	150	190	260	330
Mean	1.1	5.3	10.4	15.7	23.8	30.7	131	222	300	426	489	604
Tetracycline Hydrochloride												
E	3.5	11.9	19.5	25.3	34.2	42.9	370	640	730	830	970	1140
S	1.9	10.5	22.0	29.5	41.0	50.5	190	270	340	400	500	620
B	1.8	6.5	12.5	17.5	26.3	34.0	30	80	110	140	200	310
T	2.0	9.5	16.7	22.8	32.9	42.0	100	170	220	270	350	430
N	5.5	20.8	29.5	36.0	46.1	54.4	230	390	420	470	350	660
H	3.1	13.0	22.0	28.0	38.4	46.9	30	90	190	280	360	450
Mean	3.0	12.0	20.4	26.6	36.5	45.1	158	273	335	398	455	602
Tetracycline												
E	0.3	0.5	0.7	0.8	1.0	1.2	280	430	580	620	690	770
S	0.1	1.3	2.9	4.0	5.4	6.0	200	330	460	590	680	840
B	0.4	2.8	6.2	9.5	14.9	19.5	50	90	130	180	310	450
T	0	1.3	3.0	4.2	5.9	6.4	40	70	320	490	640	800
N	0	1.9	5.0	7.5	11.0	12.8	80	140	190	250	350	450
H	0.3	1.1	1.8	2.3	3.2	4.0	40	100	340	590	780	970
Mean	0.2	1.5	3.3	4.7	6.9	8.3	115	193	336	453	575	713

[a] All doses on the basis of 200 ± 19 mg. tetracycline hydrochloride equivalent.
[b] Bracketed numbers are subject's age in years followed by his weight in Kg.

TABLE III.—EXCRETION DATA FROM SINGLE 200-MG. DOSES OF TETRACYCLINE IN THE FORM OF SMALL PARTICLES[a]

Subject	Cumulative mg. of Tetracycline and ml. Urine Excreted to Various Times in Hours											
	mg. as Hydrochloride						Urine Volume, ml.					
	1.0	2.0	3.0	4.0	6.0	8.0	1.0	2.0	3.0	4.0	6.0	8.0
Tetracycline Hydrochloride												
E	2.5	10.4	17.4	23.0	31.9	39.4	250	570	670	790	940	1090
S	2.2	8.8	15.0	20.2	28.8	36.3	270	510	590	790	950	1100
B	1.1	6.0	13.0	21.5	32.2	39.7	30	50	70	120	220	330
T	2.2	7.2	14.2	22.5	34.8	44.3	50	80	130	180	260	350
N	0	3.3	10.4	21.0	38.3	52.0	70	150	190	230	300	370
Mean	1.6	7.1	14.0	21.6	33.2	42.3	134	272	330	422	534	648
Tetracycline Sodium Hexametaphosphate Complex												
E	2.0	8.7	17.9	24.0	33.0	40.0	270	530	590	650	760	870
S	3.0	10.0	20.2	29.5	37.9	42.5	70	150	260	380	630	920
B	3.2	8.5	15.0	22.8	35.2	45.4	50	100	150	180	250	330
T	2.2	11.2	20.5	26.0	33.4	42.0	40	80	200	250	320	390
N	0	8.0	20.2	29.4	42.2	52.6	220	550	610	690	820	920
Mean	2.1	9.3	18.8	26.3	36.3	44.5	130	282	362	430	556	686
Tetracycline												
E	2.5	9.3	16.2	21.2	28.9	34.9	320	640	770	900	1090	1180
S	2.9	8.8	17.1	27.2	46.0	59.6	170	600	790	970	1010	1220
B	2.1	6.3	11.8	17.2	25.5	32.0	70	110	130	160	240	320
T	1.0	7.2	17.9	27.0	39.4	49.0	50	100	150	200	270	330
N	1.0	5.0	11.7	20.5	36.5	48.2	600	710	780	840	910	960
Mean	1.9	7.3	14.9	22.6	35.3	44.7	242	432	524	614	704	802

[a] All doses on the basis of 200 ± 1 mg. tetracycline hydrochloride equivalent. Average diameter of particles, 100 μ.

It is not possible, in general, to obtain instantaneous excretion rates, hence average values must be used. In this paper, excretion rates are the average hourly values calculated from the slope of the mean cumulative amount *vs.* time excretion curve. For example, excretion rates at one hour represent the difference between the amounts excreted at one-half and one and one-half hours. When the interval for calculating mean rates was shortened to one-half hour, no substantial differences in values were found. The need for instantaneous rates becomes less important as the time of elimination and time for absorption increase. Tetracycline is eliminated rather slowly with a half-life in the blood stream of about eight hours (24).

Solution Rates in Vitro.—Table IV summarizes the values found with the materials and mediums used in tests. The average value shown was calculated by weighting the mean value in simulated intestinal fluids by a factor of 5. It was thought that a value weighted in this manner should more correctly represent the *in vivo* exposure of drugs to dissolution medium than a simple average of the *in vitro* rates.

TABLE IV.—*In Vitro* SOLUTION RATES[a]

Material	Simulated Mediums			
	Gastric	Neutral Intestinal	Alkaline Intestinal	Mean[b]
Tetracycline HCl	4.1	7.8	38	20
Tetracycline sodium hexametaphosphate complex	6.1	1.7	26	12
Tetracycline PSP	0.12	0.09	3.0	1.3
Tetracycline	2.6	<.001	<0.001	0.4

[a] mg./min.-cm.²
[b] See text.

Correlation Between Excretion Rate and in Vitro Solution Rate in Pellet Tests.—The cumulative amounts excreted and excretion rates found when the pelleted preparations were taken are shown in Figs. 1 and 2. An examination shows a marked difference among the preparations tested both in

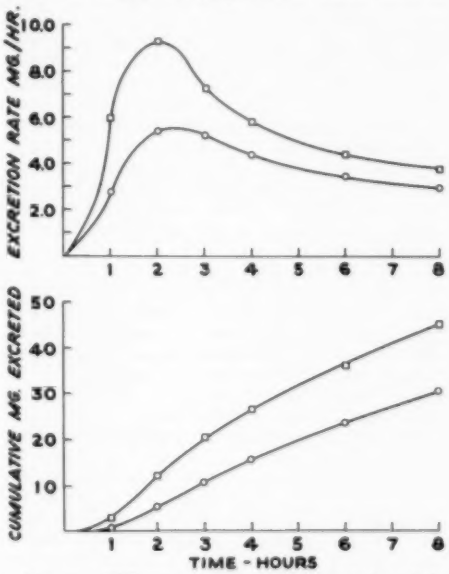

Fig. 1.—Difference in amounts excreted and in excretion rate at various times when doses of tetracycline equivalent to 200 mg. of the hydrochloride were taken in the form of pellets as the sodium hexametaphosphate complex (open circles) and as the hydrochloride itself (open squares).

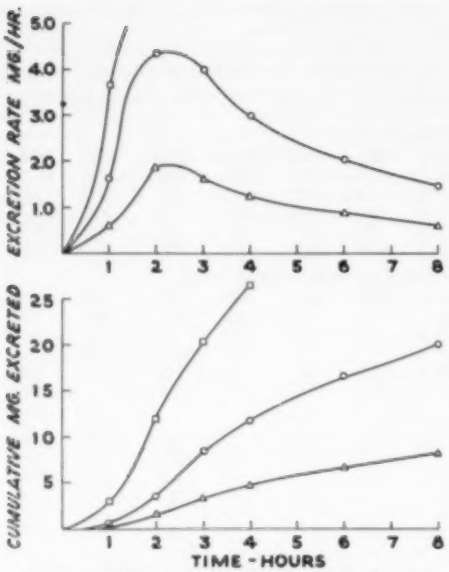

Fig. 2.—Difference in amounts excreted and in excretion rate at various times when doses of tetracycline equivalent to 200 mg. of the hydrochloride were taken in the form of pellets as the PSP salt (open circles) and as tetracycline (open triangles). Curve for the hydrochloride from Fig. 1 included for reference (open squares).

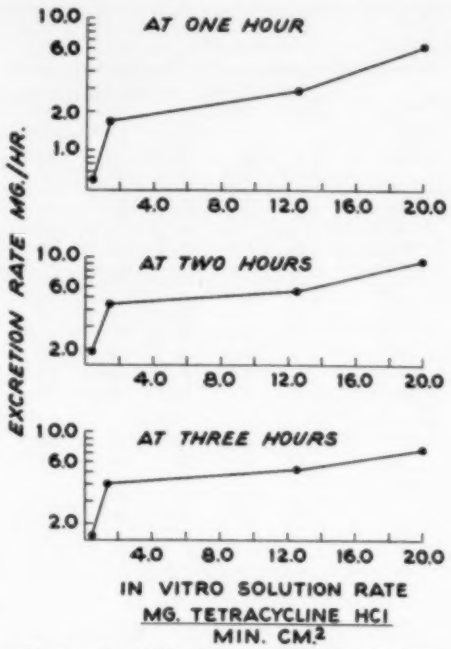

Fig. 3.—Correlation between excretion rate at one, two, and three hours and *in vitro* solution rate when various tetracyclines were taken in the form of pellets. Points, from left to right on all diagrams are, respectively, tetracycline, tetracycline PSP, tetracycline sodium hexametaphosphate complex, and tetracycline hydrochloride.

total amounts excreted and excretion rates at any given time. When the excretion rates at one, two, and three hours were plotted against the solution rates determined *in vitro*, a definite correlation was seen to exist between these quantities. This is shown in Fig. 3. This comparison is arbitrary, but it does indicate qualitatively the dependence between the quantities.

The initial surface area presented to gastrointestinal fluids when these preparations were taken was about 2.8 sq. cm. Under these conditions, there can be no doubt that *in vivo* solution rate was rate limiting in absorption.

In the curves shown on Figs. 1 and 2, the data were obtained from ten subjects in the case of the sodium hexametaphosphate complex and PSP salts and from six subjects (included in the ten) in the other cases. No substantial change in the curves resulted when the mean excreted amounts from the basic six subjects only were used. This will be discussed further, later in this report.

Excretion Rate from Small Particle Preparations.—An examination of Fig. 4 shows that when tetracyclines were given in a form where intial surface was relatively large as compared to the pellets, the mean amounts excreted and mean excretion rates were almost the same. The particles used to fill the capsules here had an average diameter of about 100 μ. On the basis that all particles were spherical with this diameter and possessed a density of 1.4, the initial surface area for tetracycline hydrochloride contained in capsules was about 85 sq. cm. For tetracycline sodium hexametaphosphate complex the value of the same quantity was about 115 sq. cm., since this material is less active than the hydrochloride salt. There was a significant difference in amounts excreted at three hours between these two preparations as will be discussed later.

Comparison of Excretion Rates from Tetracycline Salts in Pellets and as Small Particles.—The combined effects of intrinsic solution rate and initial surface area are well illustrated in Figs. 5, 6, and 7. With tetracycline, which possessed the slowest intrinsic solution rate of all the materials tested, the effect of increased surface area was extremely pronounced. Since this material dissolved quite rapidly in simulated gastric fluid, it is likely that in most cases the drug dissolved in the stomach when the capsules were taken. The necessity for large initial surface areas became less important with increase in intrinsic solution rate of the material used as is illustrated in the curves of Figs. 6 and 7, respectively, for the sodium hexametaphosphate complex and hydrochloride salts. The differences between excretion rates from pellets and capsules decreased as intrinsic solution increased. In fact, it appeared that the initial surface area needed with the hydrochloride salt was no greater than that available in the pellets of this material, since excretion rate was actually greater with pellets than with 100 μ particles. It was apparent from these results that *in vivo* solution rate was not overwhelmingly rate limiting in absorption when the hydrochloride salt was used.

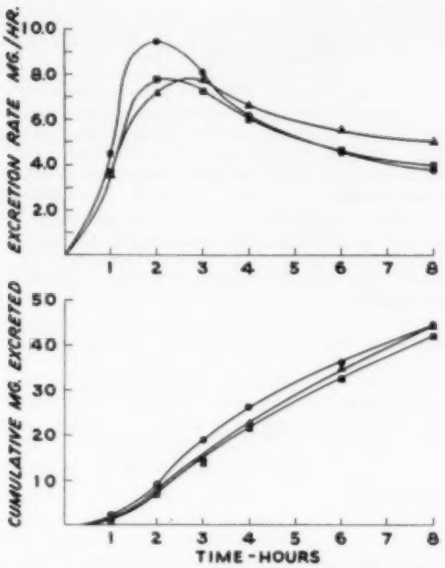

Fig. 4.—Nature of cumulative amount excreted and excretion rate curves when two tetracycline salts and tetracycline were taken in capsules containing particles with 100 μ average diameter. Solid squares—tetracycline hydrochloride, solid circles—tetracycline sodium hexametaphosphate complex, and solid triangles—tetracycline. All doses equivalent to 200 mg. of tetracycline hydrochloride.

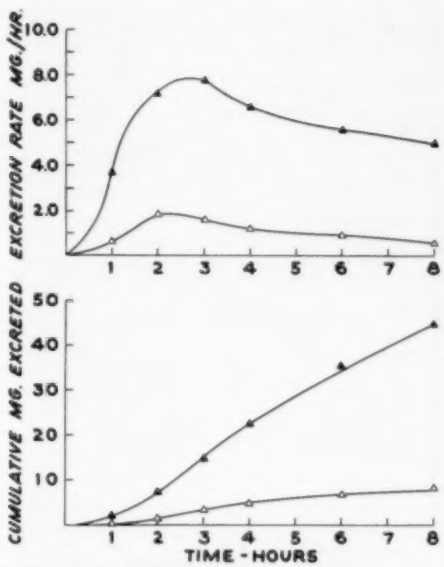

Fig. 5.—Difference in cumulative amount excreted and excretion rate curves when tetracycline was taken as pellets and as particles with average diameter of 100 μ. Dose: 200 mg. tetracycline hydrochloride equivalent. Solid triangles—tetracycline as 100 μ particles and open triangles—as pellets.

Significance of Differences in Results.—An examination of the individual entries in Tables II and III shows that relatively large variations occurred even though the means of entries indicated definite trends as has been discussed in preceding sections. The means of amounts excreted to one, two, and three hours were tested for significant differences by the *t* test and the results are summarized in Table V.

Since there was a significant difference between mean amounts excreted when pellets of the hydrochloride and sodium hexametaphosphate complex were compared, the results were significant between tetracycline hydrochloride and all the other materials used in pellets. The differences between the hydrochloride and sodium hexametaphosphate existed also in the case where the comparison was made with the basic six crossover subjects.

The differences between mean amounts excreted of tetracycline sodium hexametaphosphate complex and the PSP salts were not significant in spite of the fact that the excretion rates of these two materials were of the correct order in comparison to their *in vitro* solution rates. An examination of Fig. 3 shows, however, that the excretion rate of tetracycline PSP was much more rapid than its *in vitro* solution rate should allow when considered with the other materials. It was likely that the *in vivo* solution rate differences between these two substances were much less than that the differences indicated by the results of *in vitro* measuremets.

It might be thought that an explanation for the significant differences in mean amounts excreted at

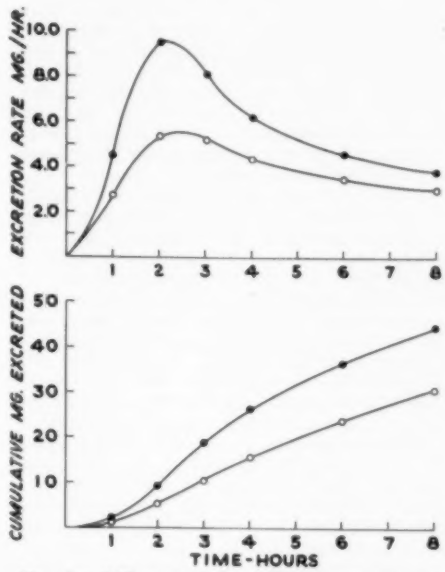

Fig. 6.—Difference in cumulative amount excreted and excretion rate curves when tetracycline sodium hexametaphosphate complex was taken as pellets and as particles with average diameter of 100 μ. Dose: 200 mg. tetracycline hydrochloride equivalent. Solid circles—100 μ particles and open circles—as pellets.

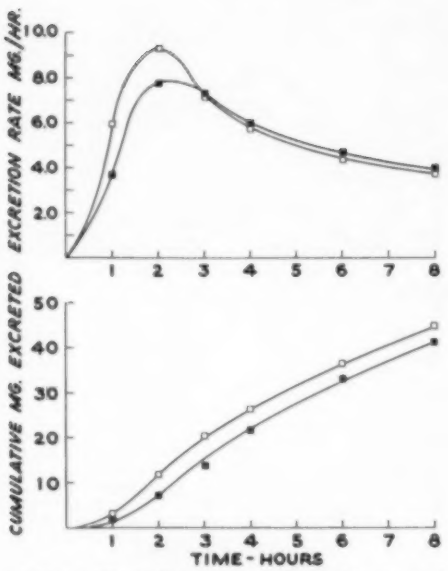

Fig. 7.—Illustrating a case, by cumulative amount excreted and excretion rate curves, where absorption of tetracycline was not rate-limited by *in vivo* solution rate. Dose in each case was 200 mg. tetracycline hydrochloride. Solid squares—as 100 μ particles and open squares—as pellets.

TABLE V.—SIGNIFICANCE OF DIFFERENCE BETWEEN MEANS OF AMOUNTS EXCRETED FROM SEVERAL PREPARATIONS (*t* TEST)

	1.0 Hour	2.0 Hours	3.0 Hours
Pellets of Tetracycline Hydrochloride and Sodium Hexametaphosphate Complex (14 Degrees of Freedom, t = 2.15[a])			
t, calculated	2.48	3.31	3.56
Pellets of Tetracycline Hydrochloride and Sodium Hexametaphosphate Complex (10 Degrees of Freedom, t = 2.23[a])			
t, calculated	1.78	2.47	2.88
Pellets of Tetracycline Sodium Hexametaphosphate Complex and PSP (18 Degrees of Freedom, t = 2.0[a])			
t, calculated	1.27	1.65	1.39
Pellets of Tetracycline PSP and Tetracycline (14 Degrees of Freedom, t = 2.15[a])			
t, calculated	1.56	3.66	2.64
Capsules of Tetracycline Sodium Hexametaphosphate Complex and Hydrochloride (8 Degrees of Freedom, t = 2.31[a])			
t, calculated	0.65	1.58	3.07
Pellets and Capsules of Tetracycline Hydrochloride (9 Degrees of Freedom, t = 2.26[a])			
t, calculated	1.95	2.00	2.34

[a] Value to be exceeded before difference between means can be considered significant (95% confidence).

three hours from small particles of the sodium hexametaphosphate complex and the hydrochloride was in the differences in the products of *in vitro* solution rate in simulated gastric fluid and surface area between the two materials. However, other results indicated that *in vivo* solution rate was not rate-limiting in absorption when capsules containing small particles were taken. Furthermore, pellets of hydrochloride gave mean rates greater than capsules of the sodium hexametaphosphate complex.

The last entry in Table V indicates the significance of the differences in mean amounts found when comparing absorption of tetracycline HCl from pellets and capsules. It seems reasonable to assume that the occurrence was due to chance.

Dose Dispersion.—The fact that tests with capsules containing relatively fine material gave nearly identical results when the hydrochloride, sodium hexametaphosphate complex, and free base were compared, does not necessarily mean that capsules containing powdered materials would always give the same results. In these tests all surface could be considered to have been made available for contact with gastrointestinal fluids due to the bicarbonate content. Addition of materials that would tend to prevent dispersion of capsule contents could be logically expected to hinder *in vivo* solution and, hence, absorption.

Sodium bicarbonate or effervescent mixtures based on sodium bicarbonate have been employed to increase the rate of absorption of sulfathiazole and acetylsalicylic acid (26). It was shown that when about 2.0 Gm. of sodium bicarbonate were given with these drugs, the rate of build-up of blood concentration with time was much more rapid than when it was omitted. It was explained that bicarbonate hastened stomach emptying and thereby transferred the drug to the intestines where it would be better absorbed. Although stomach emptying time is a factor in tetracycline absorption as a consequence of the pH-partition hypothesis (14–16), the dose used in the present work was tenfold less than the dose previously employed for increasing emptying rate (26).

The effervescence produced by contacting sodium bicarbonate and acidic gastric fluid could, on the basis of the solution rate concept advanced here, be expected to "enhance" absorption in those cases where absorption was rate-limited by surface and intrinsic solution rate. This view is reasonable when the marked effect of agitation on solution rate is considered. In fact, increased agitation seems a reasonable explanation for the sulfathiazole and acetylsalicylic acid work (26).

Solution Rate.—The differences in *in vitro* solution rate found here were due to the influence of the dissolving material on its diffusion layer. This influence has been discussed in previous publications concerning weak acids (10, 11). The situation is analogous with a material such as tetracycline even though its basic function was used in salt formation.

Tolerance to Tetracycline by Subjects.—All subjects tolerated the tetracycline dosages well. In two or three instances, slight stomach upset was noticed which could be attributed to either sodium bicarbonate or tetracycline. One subject developed a moderately severe case of urticaria after dosage

with pellets of tetracycline hydrochloride. However, continuation in the testing program by this subject after a two-week lapse of time was without further reaction to drug.

It has been reported that tetracycline taken in tablets results in more frequent side effects such as nausea, generalized itching, diarrhea, and pruritus ani than tetracycline taken in capsules (27). The present work may offer an explanation for these occurrences. Generally speaking, capsules may be considered as "high availability" dosage forms when compared to tablets. Degree of difference depends, of course, on binders, excipients, etc., used. Those side effects arising from disturbances in the gastrointestinal tract could be due to retention of undissolved drug in tablets in the intestinal tract for long periods of time. In the few tests that were made here where excretion was followed to twenty-four to twenty-eight hours, substantial amounts of pelleted preparations sometimes remained unexcreted when compared to amounts of tetracycline hydrochloride excreted in the same period. This was the case in particular with tetracycline pellets.

SUMMARY

When tetracycline absorption was studied by means of excretion rate determinations, it was found that intrinsic solution rate was rate-determining in absorption providing initial surface area was restricted in the dosage form. With increased surface area of drug in the dosage form and with provision made to make this surface available to dissolution medium *in vivo*, it appeared that overall solution rate was not rate-limiting in absorption of this drug and its salts. It was also shown that excretion rate studies were capable of supplying comparative information on the blood levels of drug.

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Studies on Glutamic Acid Decarboxylase*

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The presence of glutamic acid decarboxylase activity was demonstrated in extracts of *Papaver somniferum* and *Ricinus communis*. The product of this reaction, 4-aminobutyric acid, was found to be present in *Datura stramonium* and *Delphinium ajacis*. It was identified as the only product of glutamic acid metabolism in squash preparations and was not metabolized further.

ALTHOUGH considerable interest was aroused by the announcement of the discovery of glutamic acid decarboxylase in brain tissue of rats and mice (1–6), and in spite of the fact that this enzyme is by far the most predominant amino acid decarboxylase found in biological systems (7–11), neither its function nor the metabolic fate of the reaction product, 4-aminobutyric acid, are known at this time.

Davison (12) has offered some evidence that different enzymes are responsible for the decarboxylation of glutamic acid by rat brain and rat liver preparations.

Rogers (13) has shown that of 35 common amino acids and acids concerned with respiration only L-(+)-glutamic acid was strongly decarboxylated by acorn squash preparations. The amino group appears to be necessary for decarboxylation since 2-ketoglutaric acid is not decarboxylated under the conditions used.

Naylor and Tolbert (14) have studied glutamic acid decarboxylase in green and etiolated barley leaves. They found the rate of glutamic acid

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metabolism was faster in the light than in the dark. Through the use of isotope tracer technique they also established that 80 per cent of the glutamic acid metabolized under anaerobic conditions was decarboxylated to 4-aminobutyric acid, and that 4-aminobutyric acid was the only product *in vivo* from the action of glutamic acid decarboxylase on glutamic acid.

EXPERIMENTAL

Manometric Approach to Glutamic Acid Decarboxylase Activity.—Homogenates were prepared by mixing the fresh plant material in a Waring Blendor at 5° in the presence of an appropriate phosphate buffer and followed by filtration. The Warburg Constant Volume Respirometer was used to measure carbon dioxide evolution directly in an atmosphere of purified nitrogen according to a standard procedure (15). The bath temperature was 37.5° and the shaking rate was 116 per minute with a stroke of 3 cm.

Using the method of Rogers (13), it was found that 100 Gm. of acorn squash pulp extracted with 100 cc. of phosphate buffer of pH 5.8 gave favorable glutamic acid decarboxylase activity when a 3.0-cc. aliquot was used with 1.0 cc. of L-(+)-glutamic acid containing 33 micromoles per cc.

Applying the above conditions to whole two-month-old *P. somniferum* seedlings, a significant[1] amount of carbon dioxide evolution was observed in a set of eight experimental determinations over the controls. Glutamic acid decarboxylase activity was then observed in *R. communis* preparations of two-month-old seedlings under the same experimental conditions although it was necessary to increase the sample size to a set of 12 determinations to obtain the same level of significance. The results of these experiments are summarized in Fig. 1. *P. somniferum* was then tested under the above conditions for decarboxylase activity against substrates of DL-tryptophan, DL-phenylalanine, DL-tyrosine, and DL-dopa. No significant carbon dioxide evolution was noted.

Manometric Approach to Glutamate and 4-Aminobutyrate Metabolism.—Squash homogenates as previously described were tested for 2-ketoglutaric acid decarboxylase activity both with and without the presence of 66 micromoles of methylene blue in each reaction vessel to act as a hydrogen acceptor. No decarboxylase activity could be established in either case.

Attempts to demonstrate oxygen uptake by squash homogenates and a 4-aminobutyrate substrate were unsuccessful. It was not possible to demonstrate the formation of succinate or succinyl semialdehyde by chromatography of these reaction mixtures.

It was demonstrated by standard techniques (15) that the anaerobic decarboxylation of glutamic acid by squash homogenates exceeds the aerobic decarboxylation reaction. The system employed consisted of 1.8 cc. of the standard squash homogenate, 0.5 cc. of the standard substrate solution in an atmosphere of air for the aerobic set and in purified nitrogen for the anaerobic set. A filter paper fan and 0.2 cc. of 10% KOH were used in one flask of the aerobic set to take up the carbon dioxide evolved. This 0.2 cc. volume was equalled in all other flasks by additional buffer. Carbon dioxide evolution was observed at two different pH values; the results are summarized in Fig. 2.

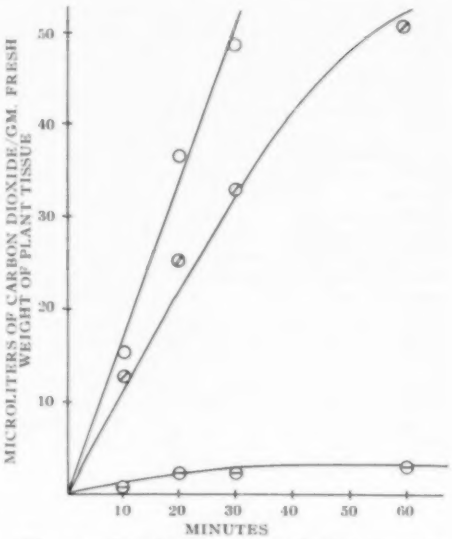

Fig. 1.—Cumulative carbon dioxide evolution from equivalent preparations of three different plant materials using an L-(+)-glutamate substrate. ○ Acorn squash, ⊕ *P. somniferum*, ⊖ *R. communis*.

Chromatographic Studies.—Paper partition chromatography was adopted in this work as a check on the manometric results. A two dimensional ascending system of *n*-butanol/acetic acid/water (4:4:1) followed by phenol saturated with water was used. The chromatograms consisted of sheets of Whatman #1 filter paper, 20 cm. square, and the material was spotted in one corner 5 cm. from each edge. The chromatograms were stapled into cylinders and placed upright on the bottoms of commercial cylindrical chromatography chambers.

For more positive identification unknowns cospotted satisfactorily in the above systems were run in *tert*-butanol/methyl ethyl ketone/water (4:4:2) followed by *tert*-butanol/methanol/water (4:5:1) in the second direction. All of the chromatograms were developed by spraying with 0.1% ninhydrin in chloroform, or, if acids of interest were suspected to be present, the ninhydrin spraying was preceded by 0.04% bromocresol green dissolved in 95% ethanol.

In the above described fashion, 4-aminobutyric acid was identified as the main reaction product with glutamic acid in acorn squash and *P. somniferum* homogenates chromatographed directly after runs in the Warburg apparatus. Free 4-aminobutyric acid was also found to be present in equivalent preparations of *R. communis*, *Datura stramonium*, and *Delphinium ajacis*.

Radioactive Tracer Studies.—2-C^{14}-labeled

[1] Criteria established at the 5% confidence level using the *t* distribution of Dixon and Massey, "Introduction to Statistical Analysis," McGraw-Hill Book Co., Inc., New York, 1951.

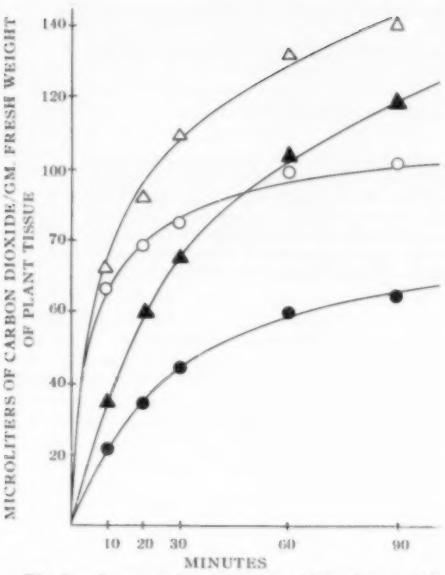

Fig. 2.—A comparison of the aerobic and anaerobic cumulative carbon dioxide evolution from acorn squash preparations at pH 5.8 and 7.4 using a L-(+)-glutamate substate. ○ Anaerobic, pH 7.4; ● Aerobic, pH 7.4; △ Anaerobic, pH 5.8; ▲ Aerobic, pH 5.8.

glutamic acid was obtained having a specific activity of 2.86 μc. per mg. Two milligrams of this material was dissolved in 1.0 cc. of a buffer of pH 5.8 and enough carrier glutamate was added to bring the concentration up to 33 micromoles per cc. This volume of substrate was run with 3.0 cc. of freshly prepared squash homogenate in a nitrogen atmosphere at 37.5°. After one hour, the reaction mixture was poured into an equal volume of boiling alcohol and filtered. Appropriate aliquots were spotted and chromatographed in the previously described manner.

Autoradiograms were prepared from the developed chromatograms and it was established that 4-aminobutyric acid was the sole reaction product from this preparation and that it was not metabolized further.

DISCUSSION

The role of 4-aminobutyric acid in metabolism and its subsequent fate has thus far successfully eluded investigators. All indications seem to point toward its being a waste product in the system employed. The Warburg studies on aerobic and anaerobic decarboxylation supports the findings of Tolbert and Naylor (14) who observed the phenomena of increased decarboxylation under anaerobic conditions in barley leaves by radioactive tracer technique. Their suggestion that the further metabolism of 4-aminobutyric acid is inhibited by anaerobiosis is given an unfavorable light by the evidence here presented that 4-aminobutyrate is not metabolized further under either aerobic or anaerobic conditions in the system employed.

We were not able to observe the decarboxylation reaction in intact tissue as repeated imbibition experiments with glutamic acid gave no results.

We wish to draw attention to the biochemical oddity demonstrated by autoradiography in that glutamic acid has a sole metabolic product in the system employed. In fact, this method was used for preparing radiochemically pure samples of 4-aminobutyric acid. Much criticism could be offered to the choice of a resting fruit for metabolism studies, but the choice was made from a viewpoint of economy, convenience, and degree of enzymatic activity. It may well be that such reactions do not occur in normal metabolism, but, if so, this work at least shows what metabolic abnormalities may occur in resting organs.

CONCLUSIONS

1. Enzyme preparation extracts from *Papaver somniferum*, *Datura stramonium*, and *Ricinus communis* were screened for amino acid decarboxylase activity under a variety of conditions and with several substrates.

2. Glutamic acid decarboxylase activity was observed manometrically in homogenates of whole seedlings of *P. somniferum* and *R. communis*.

3. 4-Aminobutyric acid was observed to be the main reaction product from glutamic acid addition to homogenates of *P. somniferum*. The presence of free 4-aminobutyric acid was established in extracts of *R. communis*, *D. stramonium*, and *Delphinium ajacis*.

4. The anaerobic decarboxylation of glutamic acid by acorn squash pulp homogenates was found to exceed the reaction under aerobic conditions at pH 7.4 and 5.8.

5. 4-Aminobutyric acid was identified as the only product of glutamic acid metabolism in squash preparations.

6. 4-Aminobutyric acid was shown not to be metabolized by squash preparations.

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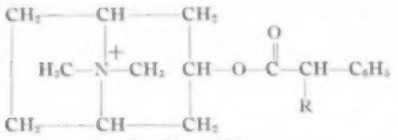
The Kinetics of the Hydrolysis of Homatropine Methylbromide and Atropine Methylbromide*

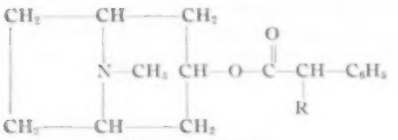
By J. L. PATEL† and A. P. LEMBERGER

A study has been made on the chemical kinetics of the hydroxyl ion catalyzed hydrolysis of homatropine methylbromide and atropine methylbromide. The temperature dependency of the hydrolytic reactions has been determined and Arrhenius equation constants are evaluated. Mandelyl tropine esters having an α-hydroxyl group are found to hydrolyze at a rate approximately five times that of the corresponding tropyl tropine esters. The positive charge in quaternary tropine esters has been shown to effect a thirty-five-fold increase in the rate of tropine ester hydrolysis.

A REPORT on the kinetics of the hydrolysis of homatropine has already been presented (1) as a part of an investigation to evaluate quantitatively the contribution of polar and steric factors and the positive ionic charge on the rate of tropine ester hydrolysis. This publication reports the kinetics of hydrolysis of homatropine methylbromide (I) and atropine methylbromide (II) and correlates the data with that previously obtained for homatropine (III) and atropine (IV) (2).



I. R = OH
II. R = CH_2OH



III. R = OH
IV. R = CH_2OH

THEORETICAL CONSIDERATION

Unlike homatropine, the mechanism of hydrolysis of homatropine methylbromide and atropine methylbromide remains the same throughout the entire pH range. Specific rate constants were therefore determined only in moderately alkaline solutions. Half-life periods for these quaternary salts can be predicted from the specific rate constants for any given hydroxyl ion concentration. The pH dependency studies were therefore not carried out. Determinations were made at temperatures ranging from 10 to 25.8°, and Arrhenius equation constants were evaluated.

Determination of homatropine methylbromide and atropine methylbromide in the presence of their degradation products is a difficult task. High water solubility of these esters limits their separation from hydrolysis products and the method used by Zvirblis, *et al.* (2), for atropine does not work. Bandelin (3) determined homatropine methylbromide by precipitating a reineckate complex, which was then dissolved in acetone and measured colorimetrically. Robinson (4) found that in the reineckate procedure, tropanol methylbromide (a hydrolysis product of homatropine methylbromide and atropine methyl bromide) yielded the same amount of color when complexed with ammonium reineckate. Robinson used Valser's reagent to assay homatropine methylbromide and reported that the absorbances of aqueous suspensions of methyl tropanol iodo-mercurate and methyl homatropine iodo-mercurate complexes differed sufficiently to allow measurement of the degree of hydrolysis of homatropine methylbromide. The typical Beer's law plot of the absorbance of the methyl homatropine iodo-mercurate complex in solution *versus* concentration as illustrated by him is not linear and the method was found to be unsatisfactory for the present investigation.

Another way of determining the bimolecular rate constants of alkaline hydrolysis of ester is to transfer aliquots from the reaction mixture into excess acid at suitable time intervals and back titrate with alkali to the appropriate end point. Garrett (5) has used the titration method for the study of the kinetics of the hydrolysis of scopolamine derivatives. For some scopolamine derivatives, rates were very high and the reaction was complete in three to four minutes. An attempt to study the hydrolysis of homatropine methylbromide by the above method showed that the ester reacted almost completely in 0.01 *N* alkali in about two minutes at room temperature. More dilute alkali was tried to reduce the rate of reaction and to facilitate the periodical removal of the aliquots from the reaction mixture. In the dilute alkali, however, the indicator end point was found to be irreproducible. Using photometric titration for the determination of substituted phenols, Goddu and Hume (6) have shown that photometric titration has an advantage over potentiometric methods when determinations are made in highly dilute solutions and with very weak acids. This led to the use of photometry, the change in absorbance of an indicator as a function of hydroxyl ion concentration, to follow the progress of the reaction. To serve this purpose, the indicator should show color changes in the selected alkali concentration, should be reasonably stable in alkali, and its acid form should not absorb

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appreciably at the absorption wavelength of the base form. Alizarin yellow R (sodium-*p*-nitrobenzene-azo-salicylate) was tried and found to fulfill the said requirements and, hence, was chosen as an indicator for the present investigation. The possibility that the ester formed a complex with the indicator seems very unlikely since the plots conformed to bimolecular kinetics.

For each indicator there is a characteristic zone of hydrogen ion concentration, on the acid side of which the indicator is completely transferred into its acid form and on the alkaline side into its base form. Within this range, there will be different proportions of the acid and base forms. If the indicator is a weak acid, its ionization can be represented by

$$HIn \rightleftharpoons H^+ + In^-$$

and the corresponding equilibrium constant is

$$K_a = \frac{(H^+)(In^-)}{(HIn)} \qquad \text{(Eq. 1)}$$

or

$$(OH^-) = K_b \frac{(In^-)}{(HIn)} \qquad \text{(Eq. 2)}$$

where K_a and K_b are the ionization constants of the acid and base form of the indicator, respectively.

Indicator base-acid ratios were calculated from its absorbance data by means of the equation

$$\frac{(In^-)}{(HIn)} = \frac{A - Aa}{Ab - A} \qquad \text{(Eq. 3)}$$

where A = absorbance during reaction; Aa = absorbance of pure acid form of indicator; and Ab = absorbance of pure base form of indicator. This approach is similar to the type III plots described by Rehm and Higuchi (7).

EXPERIMENTAL

Reagents.—Homatropine methylbromide U. S. P.; atropine methylbromide, K and K Laboratories, Inc., recrystallized from ethanol, m. p. 220–221°; approximately 0.01% alizarin yellow R in water containing 5% ethanol.

Alkaline Hydrolysis of Homatropine Methylbromide and Atropine Methylbromide.—Homatropine methylbromide was studied in solution 0.0066 *M* and 0.005 *M* in ester and 0.0066 *N* in barium hydroxide. Atropine methylbromide was studied in solution 0.0066 *M* in ester and 0.0066 *N* in barium hydroxide. Five milliliters of indicator solution and 25 ml. of barium hydroxide solution were placed in a thermostatically controlled bath and allowed to reach temperature equilibrium. A weighed amount of ester was then added and rapidly mixed with the solution. The reaction mixture was periodically transferred into the cell of a Cary model 11 recording spectrophotometer and the absorbance at wavelength 493 mμ was recorded. For room temperature studies, the reaction was allowed to take place in the spectrophotometer cell after the first transfer. Studies were made at 10, 15, 20, and 25.8°, to allow calculation of the Arrhenius constants.

Absorbance of the solution containing 5 ml. of indicator solution and 25 ml. of barium hydroxide solutions of varying strengths served as a blank to calculate hydroxyl ion concentration during the course of reaction. All the determinations were made in an atmosphere of nitrogen.

RESULTS AND DISCUSSION

The kinetics of the alkaline hydrolysis of the ester groups in homatropine methylbromide and atropine methylbromide were determined by estimation of the slopes appropriate to the bimolecular rate expressions

$$\frac{1}{a - x} = kt + \text{constant} \qquad \text{(Eq. 4)}$$

$$\log \frac{b}{a} \frac{a - x}{b - x} = \frac{kt(a - b)}{2.303} \qquad \text{(Eq. 5)}$$

Thus $\frac{1}{a-x}$ was plotted against the time when $a = b$ and $\log \frac{b}{a} \frac{a-x}{b-x}$ was plotted against time when $a \neq b$ where a and b are the initial concentrations and $(a - x)$ and $(b - x)$ are the concentrations at time intervals for the alkali and ester, respectively. Hydroxyl ion concentration was calculated from the absorbance data and ionization constant of the indicator using Eqs. 2 and 3. Calculation of the ester concentration was based on the postulated 1:1 reaction of ester with hydroxide ion. A typical blank plot showing the validity of Eq. 2 is shown in Fig. 1. Experimentally determined ionization constants of the base form of the indicator are given in Table I.

As illustrated in Figs. 2 and 3, the observed rate of hydrolysis of homatropine methylbromide and atropine methylbromide is second order. The bimolecular rate constants (k) in liter mole^{-1}second^{-1} are given in Table II.

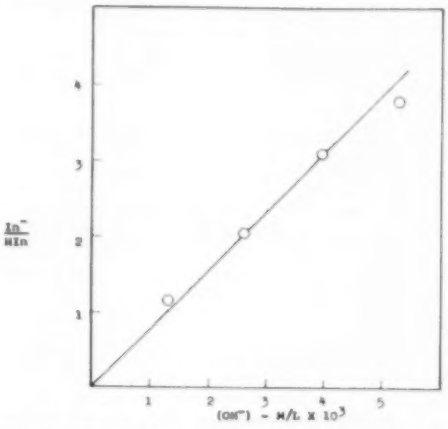

Fig. 1.—Blank plot relating the indicator absorbance with the hydroxyl ion concentration at 25.8°.

TABLE I.—IONIZATION CONSTANTS OF ALIZARIN YELLOW R, BASE FORM

Temp. °C.	$K_b \times 10^3$
10.0	1.10
15.0	1.15
20.0	1.22
25.8	1.31

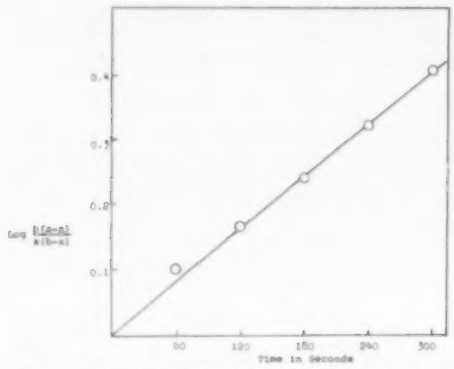

Fig. 2.—Typical bimolecular rate plot for the alkaline hydrolysis of homatropine methylbromide at 25.8°.

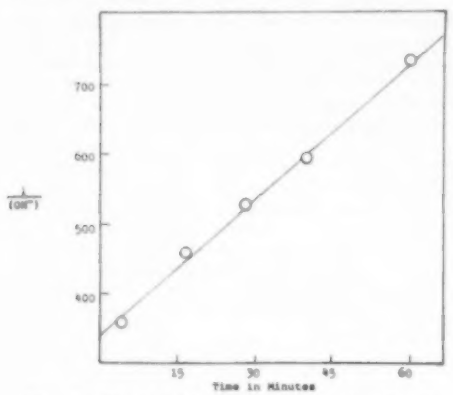

Fig. 3.—Typical bimolecular rate plot for the alkaline hydrolysis of atropine methylbromide at 10°.

TABLE II.*—BIMOLECULAR RATE CONSTANTS FOR ALKALINE HYDROLYSIS OF HOMATROPINE METHYLBROMIDE AND ATROPINE METHYLBROMIDE

Temp. °C.	k in liter mole^{-1} sec.$^{-1}$ Homatropine Methylbromide	Atropine Methylbromide
10.0	0.53	0.11
15.0	0.78	0.17
20.0	1.11	0.26
25.8	1.74	0.37

* Calculated bimolecular rate constants in liter mole^{-1} sec.$^{-1}$ for homatropine and protonated homatropine at 25.8° are 0.039 and 1.54, respectively (1).

Temperature Dependence.—Rate constants for homatropine methylbromide and atropine methylbromide were determined at four different temperatures. Bimolecular rate constants are plotted against the reciprocal of the absolute temperature in Figs. 4 and 5. The activation energy was calculated by setting the slope equal to $-E/2.303R$ and the frequency factor (s) in the Arrhenius equation was calculated using $k = se^{-E/RT}$. The constants of the Arrhenius equation are given in Table III.

A significant difference in the specific rate constants is observed when the values for various tropine esters are compared. Ingold (8) has shown the retarding effect of alkyl substituents and negative ionic charge and the accelerating influence of electron attracting Cl, OH, $COCCH_3$, $COCH_3$, CH_2COCH_3, and CH_2OCH_3 groups on the alkaline hydrolysis of aliphatic carboxylic esters. The difference in rates has been attributed by this author clearly to the polar effects, whatever the steric effects may be. Garrett (5) in his studies on the kinetics of scopolamine derivatives has attributed the increased rates in positively charged quaternary salts to the field effects.

In the case of tropine esters, it is found that mandelyl tropine esters, *viz.* base and acid form of homatropine and homatropine methyl bromide, degrade at a rate approximately five times that of the corresponding tropyl tropine esters, *viz.* the base and acid form of atropine and atropine methyl bromide. The accelerating effect of the α-hydroxyl group in mandelyl tropine esters is in agreement with the scheme postulated by Waters (9) and Ingold and Ingold (10) to explain the alkaline hydrolysis of esters.

TABLE III.—TABULATION OF CONSTANTS OF THE ARRHENIUS EQUATION

Compound	E Kcal./Mole	Log S*
Homatropine methylbromide	13.0	9.74
Atropine methylbromide	14.0	9.82

* Log $k = -E/2.303\,RT + \log S$ where k is in liter mole^{-1} sec.$^{-1}$.

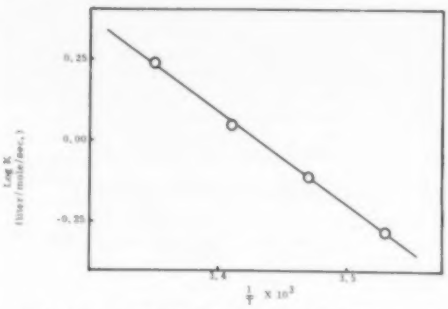

Fig. 4.—Arrhenius plot of the bimolecular rate constants for the alkaline hydrolysis of homatropine methylbromide.

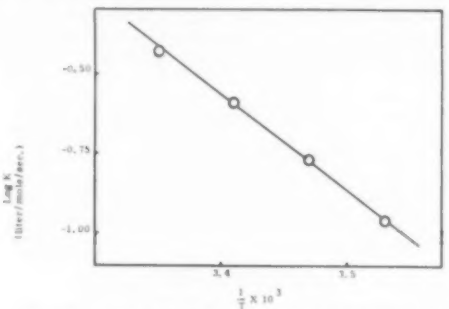

Fig. 5.—Arrhenius plot of the bimolecular rate constants for the alkaline hydrolysis of atropine methylbromide.

Comparison of the rate constants of protonated homatropine, homatropine methylbromide, and atropine methylbromide with the corresponding free bases has shown that positive charge on nitrogen in quaternary salts enhances their rate very significantly. The 35-fold increase in the rates in these quaternary tropine esters may be attributed to the field effects resulting in increased concentration of hydroxide ion in the neighborhood of the ester molecule.

Thus, it is observed that the polar and/or steric effects have a great influence in determining the rate of alkaline hydrolysis of highly substituted esters such as tropine esters.

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The Effect of Adipic Acid and Methylamine on Alkaloid Biosynthesis in Certain Members of the *Solanaceae* I*

Datura stramonium Linné

By M. S. BROOKS, L. A. SCIUCHETTI,† and D. P. N. TSAO

Adipic acid administered by injection in the concentration of 0.5 per cent increased the total alkaloids (calculated as hyoscyamine) in the leaves (and flowering tops), stems, and roots by 14, 26, and 79 per cent, respectively. Methylamine in the same concentration increased the total alkaloid yield in the leaves and roots by 10 and 39 per cent while a 12 per cent decrease was observed in the total alkaloids of the stems. The plants treated with adipic acid and methylamine displayed an increase of alkaloid yields in the leaves and roots by 22 and 65 per cent while no change in alkaloid production in the stem was observed.

ROBINSON'S method (1) of synthesis of tropine has been applied to the synthesis of a number of tropinone-like compounds by replacing the succinic dialdehyde with various aldehydes such as adipic dialdehyde (2). Adipic acid was found in the juice of the sugar beet by Vavruch (3). Kameda and Toyoura (4) reported that adipic acid has been utilized by soil bacteria as a source of carbon in culture media. Dagley and Rodgers (5) have reported that intact cells of a certain vibrio grown at the expense of adipic acid gave high initial rates of oxidation of citrate. Since adipic acid and methylamine have been proposed as starting materials for the biosynthesis of tropine by Trautner (6), it was deemed desirable to study the influence of these two chemicals on alkaloid biosynthesis in *Solanaceous* plants, especially *Datura stramonium*.

EXPERIMENTAL

Procedure.—Stramonium plants employed in this study were grown under carefully controlled greenhouse conditions.[1] Seeds were germinated in culture flats containing soil composed of two parts of compost and one part of peat moss on December 29, 1955. Sixty seedlings were transplanted into pots on June 26, 1956. On August 21, 1956, twenty-four plants of approximately the same height and in similar states of growth were selected for this study and divided into four groups. Each group consisted of six plants. One group was designated as control or untreated. The remaining three groups were treated with 0.5% solutions of adipic acid, methylamine HCl, and a combination of adipic acid and methylamine HCl. Each plant of the treated groups received 3–4 ml. of the respective solutions at a rate of about 2 ml. per hour by injection into the stem (7). Two injections were given to each plant of the treated group. The plants of the control group received no injection. The first injection was administered on August 21, 1956. A second injection was given on the following day. No observable toxic effects were noted in the treated plants.

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This study has been supported in part by funds from General Research of the Graduate School of Oregon State College.
This paper was presented to the A. PH. A. Convention, Los Angeles Meeting, April 1958.
† Present address: Pharmacognosy Department, School of Pharmacy, Oregon State College, Corvallis, Ore.

[1] The authors are indebted to Dr. W. R. Naumann and Mr. M. Bakke of Drug Plant Gardens and Laboratories, College of Pharmacy of the University of Washington, for their generous assistance during the growth of Datura plants and injection procedure.

TABLE I.—TOTAL ALKALOIDS OF *Datura stramonium*

Group	Leaves		Stems		Roots	
	Alkaloids,[a] %	Difference, %	Alkaloids,[a] %	Difference, %	Alkaloids,[a] %	Difference, %
Control	0.230	..	0.058	..	0.071	..
Adipic Acid 0.5%	0.263	+14.3	0.073	+25.9	0.127	+78.9
Methylamine HCl 0.5%	0.253	+10.0	0.051	−12.1	0.099	+39.4
Adipic Acid 0.5% and Methylamine HCl 0.5%	0.281	+22.2	0.058	+00.0	0.117	+64.8

[a] Alkaloids calculated as hyoscyamine.

The plants were harvested on August 23, 1956. No significant difference was noted in the total weight of the groups receiving treatments compared with the control group. The plants were divided into three portions: leaves (including flowering tops), stems, and roots. The fresh materials were then transferred immediately into a hot-air circulating dryer and dried at a temperature of approximately 49°. The dried material was ground into a No. 60 powder and stored in an airtight container until subsequent alkaloid analyses.

The Total Alkaloid Assay.—The dried leaves, stems, and roots were assayed for total alkaloids according to the Witt and Youngken method (8) modified by using chloroform in place of benzene as the immiscible solvent. This study indicated, in general, that adipic acid and/or methylamine in the concentration of 0.5 per cent increased the total alkaloid yield (calculated as hyoscyamine) in the various plant organs (see Table I and Fig. 1). The figures represent the average of four assays for each group.

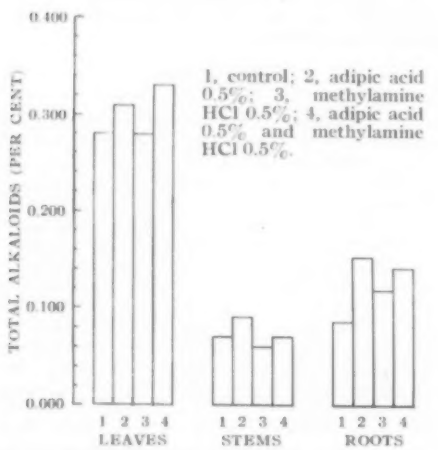

Fig. 1—Total alkaloid yield of *Datura stramonium*.

DISCUSSION

The data on the total alkaloids (Table I and Fig. 1) revealed that adipic acid in the concentration of 0.5 per cent stimulated alkaloid formation in the leaves, stems, and roots by 14, 26, and 79%, respectively. Methylamine in the same concentration increased alkaloid production in the leaves and roots by 10 and 39%, while a decrease of 12% in total alkaloids was found in the stems. The combination of adipic acid and methylamine increased total alkaloids in the leaves and roots by 22 and 65%, while no change in alkaloid formation in the stems was observed. In general, increased production of total alkaloids in the various plant organs resulted from the treatments. Adipic acid appeared to have the greatest influence. Mothes (9) has suggested that diketoadipic acid was a precursor of the *Solanaceous* alkaloids. This study showed that adipic acid stimulated alkaloid biosynthesis in *Datura stramonium* and would tend to confirm his work since adipic acid and diketoadipic acid are chemical homologues.

SUMMARY

1. Adipic acid, 0.5 per cent, increased the total alkaloid yield in the leaves, stems, and roots.

2. Methylamine increased total alkaloids in the roots, but there was no great difference in total alkaloid yield in the leaves and stems.

3. The combination of adipic acid and methylamine showed an increase of the total alkaloids in the leaves and roots, but not in the stems.

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Analysis of Phenylephrine and Tetracaine by Filter Paper Chromatography*

By HERBERT SCHRIFTMAN

Experimental preparations containing phenylephrine hydrochloride and tetracaine hydrochloride have been assayed by a paper chromatographic procedure. The results have compared favorably to those obtained by present spectrophotometric and colorimetric methods. Deteriorated samples, obtained from stability studies, have been shown by present methods to contain almost all the phenylephrine hydrochloride as claimed. However, paper chromatographic assays of these same samples have shown the phenylephrine content to be below claim and one to three degradation compounds to be present. For the chromatographic assay, a normal butanol-acetic acid-water (5:1:3) solvent system is employed and a spray reagent containing diazotized *p*-sulfanilic acid is used to detect the phenolic compounds on the paper. The completed chromatograms are quantitatively analyzed by photoelectric densitometry. Investigations are being conducted to identify the decomposition products.

PHENYLEPHRINE HYDROCHLORIDE, a sympathomimetic amine has been in general use a long time but is becoming available in a growing number of pharmaceutical preparations, containing other active compounds. These complex mixtures present the analyst with additional laborious filtering and extraction steps in conducting successful assays with the conventional colorimetric or spectrophotometric methods. Specifically, one of these methods is Auerbach's colorimetric procedure (1) which is based on the intensity of the azo color produced by coupling phenylephrine with diazotized *p*-nitroaniline. But this reaction only indicates the presence of the hydroxyphenol nucleus in the phenylephrine molecule. Hence, when the various experimental mixtures of phenylephrine hydrochloride in combination with other active compounds are subjected to stability studies, any changes on the side chain would not be detectable by this method and the preparation would apparently still contain all the phenylephrine as claimed. Ellin and Kondritzer (2) presented a colorimetric and spectrophotometric method for analyzing solutions containing phenylephrine hydrochloride and tetracaine hydrochloride. But their color reaction for phenylephrine is also based on the presence of the hydroxyphenol group. They treated the phenylephrine hydrochloride solutions with 15 per cent $HgSO_4$ and then diazotized with 0.1 per cent $NaNO_2$. Phenols and phenolic derivatives are known to react with mercuric salts in the presence of nitrous acid to form intensely colored compounds. The main drawbacks in their procedures were: (*a*) the formation of a precipitate on the addition of the $HgSO_4$ reagent when glycols are present in the solution and (*b*) the additional step in their assay of having to extract any *p*-butylaminobenzoic acid that may be present, as this compound is known to be a hydrolysis product of tetracaine. In the paper partition chromatographic procedure presented here, the extraction and filtering processes are eliminated and separate quantitative analyses of these compounds are possible.

EXPERIMENTAL

Standard mixtures of the hydrochloride salts of tetracaine and phenylephrine in 5 to 25 μg. amounts were spotted and then separated out on Whatman No. 1 chromatographic paper sheets by the upper phase of a butanol-acetic acid-water (5:1:3) solvent system, using the descending technique for twenty hours. The tetracaine salt, analogous in structure to alkaloids in that it is a basic nitrogenous compound, might be expected to react to a modified Dragendorff spray reagent, as reported by Munier (3) for detecting various alkaloids that he had separated out on paper. This reagent consisted of a mixture of bismuth subnitrate in glacial acetic acid and KI solution. The reagent was prepared from two solutions: solution 1 contained 0.85 Gm. of bismuth subnitrate dissolved in 40 ml. of water and 10 ml. of glacial acetic acid; solution 2 contained 8 Gm. of KI in 20 ml. of water. Just before use, 5 ml. each of solutions 1 and 2 were mixed with 20 ml. of glacial acetic acid and 100 ml. of water. The tetracaine spots, when sprayed with this reagent, gave the characteristic alkaloid reaction—a red spot on a light orange background. The average R_f value of the tetracaine spots was 0.83. For the separated phenylephrine hydrochloride spots, a general spray reagent for phenols was used, diazotized *p*-sulfanilic acid (4). The diazo reagent was prepared by mixing 20 ml. of a 5% $NaNO_2$ solution with 20 ml. of a solution containing 1 Gm. of *p*-

* Received May 10, 1958, from Wyeth Laboratories, Radnor, Pa.

This work was begun in the Physiology Division of the Chemical Warfare Lab., Army Chemical Center, Md.

A portion of this paper was presented at the Pharmacy Section meeting of the A.A.A.S., December 26, 1957, in Indianapolis, Ind.

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sulfanilic acid and 15 ml. of concd. HCl in a liter of distilled water. After spraying with this diazo reagent, the paper was made alkaline with a 5% K_2CO_3 spray. The phenylephrine hydrochloride salts showed up as intense yellow spots whose average R_f value was 0.63. Determination of these R_f values enabled us to cut the developed paper, prior to spraying, so that phenylephrine and tetracaine hydrochloride spots could be analyzed separately. All the colored spots were quantitatively analyzed with a Photovolt Densitometer, Model 525, containing semiautomatic plotting equipment and color filters. Table I shows that for the concentration range studied, the total color density readings are directly proportional to the concentration of the tetracaine and phenylephrine hydrochloride salts. Table II lists the results obtained from the analyses of powdered mixtures of these drugs which had been subjected to stability studies. The results obtained by the chromatographic method compare favorably with those obtained by the spectrophotometric and colorimetric method as described by Ellin and Kondritzer (2). However, when solutions of these powders are subjected to heat, these colorless solutions turn brown and paper chromatographic analysis discloses the presence of another phenolic spot at R_f 0.57, as well as other less intense phenolic spots at various R_f values. Assays of these deteriorated samples by the Ellin and Kondritzer method indicated that they contained almost all the phenylephrine hydrochloride as claimed. Quantitative paper chromatography of these same samples disclosed that the phenylephrine content was below claim in the range of 12 to 28% depending on the temperature conditions. Table III illustrates some preliminary observations and R_f values of various degradation products of phenylephrine after undergoing accelerated storage conditions. Further studies on the isolation and characterization of the various breakdown products of phenylephrine are under way and will be reported at a later date. Initial absorption spectra of the main breakdown product as well as two other degradation products has been obtained and are compared to the absorption spectra of phenylephrine hydrochloride in Fig. 1. All curves in Fig. 1 were obtained on a Beckman Recording Spectrophotometer. The concentration of the phenylephrine hydrochloride solution was 0.05 mg./ml. in absolute alcohol. The other curves represent eluates of spots of various R_f values from ten papergrams containing ten 200 μg. spots of a degradated solution of 5 mg./ml. of phenylephrine in 5% dextrose (see Table III). The curve for the

TABLE I.—RELATIONSHIP BETWEEN CONCENTRATION AND TOTAL COLOR DENSITIES OF PHENYLEPHRINE HCl AND TETRACAINE HCl SPOTS OBTAINED BY PAPER PARTITION CHROMATOGRAPHY

	μg.	Total Color Density = Area under Curve in mm.²	Density/Concn. = K
Tetracaine HCl, using a 530 mμ color filter	5	24.0	4.8
	10	48.0	4.8
	15	71.5	4.8
	20	93.5	4.7
	25	118.0	4.7
Average			4.8
Phenylephrine HCl, using a 440 mμ color filter	5	230	46
	10	449	45
	15	680	45
	20	939	47
	25	1119	45
Average			46

TABLE II.—COMPARISON BETWEEN PAPER CHROMATOGRAPHIC AND SPECTROPHOTOMETRIC METHODS USED IN THE ANALYSIS OF POWDERS OBTAINED FROM PACKETS STORED UNDER VARYING TEMPERATURE CONDITIONS

Samples	Analysis for Tetracaine HCl, mg. C[a]	S	Analysis Phenylephrine HCl, mg. C	S
1—Blank	0	0	0	0
2—Standard	25.0	25.0	25.0	25.0
3—Room Temp.	27.0	27.0	29.0	28.5
4—51°	38.5	38.9	31.5	33.0
5—65°	33.3	33.5	29.6	31.0
6—70°	32.5	31.5	31.5	30.5

[a] C = Chromatographic. S = Spectrophotometric.

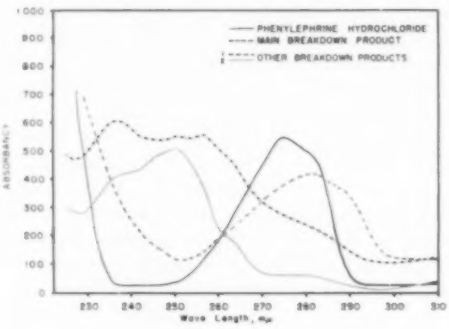

Figure 1.

TABLE III.—R_f VALUES OF PHENYLEPHRINE HYDROCHLORIDE AND ITS DEGRADATION PRODUCTS PRESENT IN DIFFERENT SOLUTIONS, AFTER STORAGE AT 75° C. FOR ONE WEEK

Solution	R_f	Reagent: Fluorescence[a]	Phenolic[b]	Secondary N[c]
5 mg./ml. in distilled water	0.57	+	+	—
	0.64	+	+	+
	0.74	+	+	...
	0.86	+	—	...
5 mg./ml. in 50% glycerin	0.57	+	—	...
	0.59	+	+	—
	0.62	+	+	+
	0.71	+	+	...
5 mg./ml. in 5% dextrose	0.26	+	—	...
	0.37	+	—	...
	0.58	+	+	—
	0.64	+	+	+
	0.75	+	—	...

[a] Fluorescence observed under U. V. light, long-wave type.
[b] Diazotized *p*-sulfanilic acid reagent + 5% K_2CO_3 as described in text.
[c] 0.2% ninhydrin in 96% ethanol, containing 2% of acetic acid and 0.5% of cadmium acetate, a positive reaction is the presence of a pink spot (5).

main breakdown product represented eluates of spots at R_f 0.58, while the U. V. spectrum for breakdown products Nos. 1 and 2 were obtained from spots at R_f 0.26 and 0.37, respectively. In all cases, 35 ml. of absolute alcohol was used for eluting the spots from the chromatograms, and then concentrated to 5 ml. prior to scanning. The reference solution was prepared from a similarly treated blank papergram.

SUMMARY

1. A paper chromatographic method is employed for analyzing phenylephrine hydrochloride and tetracaine hydrochloride which compares favorably with the spectrophotometric method.

2. The method presented here for the analysis of phenylephrine HCl and tetracaine HCl is recommended when coloration, foreign matter, or precipitants are present in the solutions.

3. Preliminary studies have been conducted to isolate and identify the degradation compounds of phenylephrine.

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Inhibition of Glucose-6-phosphatase Activity by Hypoglycemic 2-Benzenesulfonamido-5-alkyl-1,3,4-thiadiazoles*

By ROLAND JASMIN and WILLARD JOHNSON

Inhibition of glucose-6-phosphatase (G-6-P-ase) has been considered as a possible mechanism of action of the hypoglycemic sulfonylurea compounds. Experiments have been carried out to ascertain if the hypoglycemic activity of these agents could be correlated with their effect on G-6-P-ase activity. The latter was determined by the method of Cori and Cori (*J. Biol. Chem.*, 199, 661(1952)). A number of newly synthesized 2-benzenesulfonamido-5-alkylthiadiazoles which inhibited G-6-P-ase activity completely at 2.5 or 5×10^{-3} *M* had negligible hypoglycemic effect, as compared with tolbutamide, which at 8×10^{-3} *M* gave only 50 per cent inhibition and was strongly hypoglycemic. Sulfaethylthiadiazole, an antibacterial sulfonamide with no hypoglycemic activity, inhibited G-6-P-ase activity to the same extent as did tolbutamide. Phenethyldiguanide (PEDG), a nonsulfonylurea compound with marked hypoglycemic activity, failed at 5×10^{-3} *M* concentration to inhibit G-6-P-ase. The evidence indicates that no relationship exists between hypoglycemic activity *in vivo* and G-6-P-ase activity *in vitro*.

THERE IS EXTENSIVE EVIDENCE to support the view that the liver is an important, if not the principal, site of action of the oral diabetic compounds (1–4), the activity of these compounds in the hepatectomized animal notwithstanding (5). Thus, Anderson, *et al.* (1), observed in dogs a 33 per cent decrease in glucose output of the hepatic vein within ten minutes of intravenous tolbutamide administration. A similar effect of tolbutamide was noted by Ashmore, *et al.* (2), in dogs and rats, and by Kibler and Gordon (3) in diabetic patients.

The liver is virtually the sole source of blood sugar during the postabsorptive state. Directly involved in the release of glucose to the blood stream is the enzyme, glucose-6-phosphatase, which catalyzes the conversion of glucose-6-phosphate to glucose and inorganic phosphate. Cori and Cori (6) have found this enzyme to be almost completely absent from the livers of human cases of glycogen storage (von Gierke's) disease.

Inhibition of glucose-6-phosphatase has been considered as a possible explanation for the decreased output of glucose by the liver following the administration of hypoglycemic sulfonamides. Some investigators have demonstrated an inhibitory effect by tolbutamide and carbutamide under various experimental conditions (7–10). Glucose-6-phosphatase as a primary site of action of these substances has been largely discounted due to the fact that the concentrations required to inhibit the enzyme *in vitro* are much greater than the amounts needed for a hypoglycemic response in the intact animal.

The synthesis of a large series of 2-benzenesulfonamido-5-alkyl-1,3,4-thiadiazoles in our laboratories (11) enabled us to study the relationship between the inhibitory effects of these compounds

* Received June 30, 1958, from the Frank W. Horner Ltd., Research Laboratories, Montreal, Quebec.

on glucose-6-phosphatase *in vitro* and their hypoglycemic activity. It was considered that, apart from contributing to the elucidation of the mechanism of action of these agents, the establishment of such a correlation would provide a valuable screening method for hypoglycemic agents.

EXPERIMENTAL

Albino mice (20 to 30 Gm.) fed *ad libitum* on Purina Fox Chow were used in these studies. The animals were killed by decapitation, and the livers rapidly removed and placed on ice. Liver homogenates (20% w/v) were prepared in cold distilled water. The contents of each experimental vessel was as follows: 0.5 ml. of 0.02 *M* glucose-6-phosphate; 0.2 ml. of 0.3 *M* potassium citrate buffer, pH 6.8; 0.3 ml. of 20% liver homogenate; 1 ml. of glucose-6-phosphatase inhibitor to give a final concentration as indicated in the tables. The control vessel contained 1 ml. of water in place of inhibitor. Incubation was for thirty minutes at 32° with constant shaking. The glucose-6-phosphatase activity was determined by the method of Cori and Cori (6). The release of inorganic phosphate was measured by the method of Fiske and Subba Row (12).

Glucose-6-phosphate, as the barium salt, was purchased from Schwartz Laboratories Inc., and was converted to the sodium salt before using. Neutral solutions of the sodium salts of the sulfonamidothiadiazoles and of Mobenol (tolbutamide, Horner) were used in all studies. The basic structure of the thiadiazole derivatives is shown in Table I.

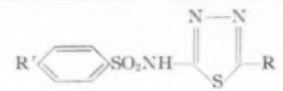
RESULTS AND DISCUSSION

The inhibitory effects of the sulfonamidothiadiazole derivatives and other hypoglycemic agents on mouse liver glucose-6-phosphatase are given in Tables I and II. The relative hypoglycemic activities of these substances in the rabbit, which have been reported by McColl, *et al.* (13), from this laboratory, are roughly indicated in the last column. It should be noted that the dosage employed to obtain the indicated hypoglycemic response bears no relation to the concentration of substance at which glucose-6-phosphate was tested.

It may be seen from Table I that many of the substituted thiadiazoles (compounds, 3, 5, 6, 13, 15, and 17) which inhibited glucose-6-phosphatase completely at 2.5 and 5×10^{-3} *M* had insignificant hypoglycemic activity *in vivo*, as compared with Mobenol (tolbutamide, Horner), which at 5×10^{-3} *M* concentration inhibited glucose-6-phosphatase by 37.5% and had strong hypoglycemic activity. The thio analog of Mobenol, devoid of hypoglycemic activity, was slightly more active than Mobenol as an inhibitor of glucose-6-phosphatase. Phenethyldiguanide, which has been shown to be a potent hypoglycemic agent in the normal and alloxanized animal (14) failed completely to inhibit glucose-6-phosphatase, as did glycocyamine (guanidoacetic acid).

Table II provides a comparison of the sulfonamidothiadiazoles in terms of the concentrations required for 50% inhibition of glucose-6-phosphatase. Here it may be seen that compounds 7 and 8, which were found to have potent hypoglycemic activity, inhibited glucose-6-phosphatase to the same extent

TABLE I.—PER CENT INHIBITION OF MOUSE LIVER GLUCOSE-6-PHOSPHATASE



Compd.	Substitution R′	Substitution R	Molar Concentration 5×10^{-3}	Molar Concentration 2.5×10^{-3}	Molar Concentration 1.25×10^{-3}	Hypoglycemic Activity[a]
		A. Sulfonamidothiadiazoles				
1	CH_3O	*t*-butyl	–	–	20	+++
2	CH_3	*t*-butyl	–	98	32	++
3	C_2H_5O	*t*-butyl	–	100	21	+
4	CH_3O	*n*-butyl	–	100	–	++
5	CH_3	*n*-butyl	–	100	–	+
6	C_2H_5O	*n*-butyl	–	100	–	+
7	CH_3O	iso-butyl	–	–	34	+++
8	CH_3	iso-butyl	–	–	54	+++
9	C_2H_5O	iso-butyl	–	93	–	–
10	CH_3O	*n*-amyl	–	–	29	+++
11	CH_3	*n*-amyl	–	–	98	++
12	CH_3O	iso-amyl	–	–	26	+
13	CH_3	iso-amyl	–	–	100	+
14	CH_3O	iso-propyl	100	–	–	++
15	CH_3	iso-propyl	100	–	–	–
16	CH_3O	*n*-propyl	44	–	–	–
17	CH_3	*n*-propyl	100	–	–	–
		B. Miscellaneous				
	Mobenol		37.5	–	–	+++
	Thio analog of Mobenol		51.0	–	–	–
	Phenethyldiguanide		0.0	–	–	+++
	Glycocyamine		0.0	–	–	++

[a] +++ indicates a decrease in blood glucose of 30–40 mg. %; ++, 20–29; +, 10–19; –, less than 10.

TABLE II.—CONCENTRATION OF SUBSTANCE REQUIRED FOR 50% INHIBITION OF GLUCOSE-6-PHOSPHATASE

Compd.	Sulfonamidothiadiazole R′	R	Moles/Liter	Hypoglycemic Activity[a]
1	CH_3O	*t*-butyl	3.0×10^{-3}	+++
2	CH_3	*t*-butyl	2.0×10^{-3}	++
4	CH_3O	*n*-butyl	1.5×10^{-3}	++
7	CH_3O	iso-butyl	2.25×10^{-3}	+++
8	CH_3	iso-butyl	1.5×10^{-3}	+++
11	CH_3	*n*-amyl	6.0×10^{-4}	++
17	CH_3	*n*-propyl	2.0×10^{-2}	−
18	C_2H_5O	*n*-propyl	2.0×10^{-3}	−
19	NH_2	ethyl	8.0×10^{-2}	−
	(sulfaethylthiadiazole)			
	Mobenol		8.0×10^{-3}	+++

[a] See Table I.

as compounds 17 and 18, which have no hypoglycemic activity. Similarly, Mobenol and sulfaethylthiadiazole, a potent antibacterial sulfonamide, each inhibited glucose-6-phosphatase by 50% at 8.0×10^{-3} *M*, while only the former had hypoglycemic activity.

CONCLUSIONS

These results clearly show the complete absence of any correlation between the hypoglycemic effects of these compounds and their glucose-6-phosphatase inhibitory effects *in vivo*. They tend to confirm the observations of other investigators that inhibition of glucose-6-phosphatase is not the principal mechanism by which these compounds exert their effects. That is not to say, however, that inhibition of glucose-6-phosphate *in vivo* does not, in some cases, contribute to the overall hypoglycemic effect.

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The Effect of Nicotine on Hypercholesterolized Cockerels*

By DUANE G. WENZEL, JAMES A. TURNER, and DONALD KISSIL

Cockerels were hypercholesterolized with a 1 per cent cholesterol diet and administered nicotine (2.28 milligrams per kilogram per day) in the drinking water. After sixteen weeks of treatment, the plasma cholesterol and lipid phosphorus levels and the cardiovascular pathology were not significantly different from those of the cholesterol controls.

A NUMBER of recent reports have suggested a possible causal relationship between nicotine and atherosclerosis. Mortality studies reveal a twofold increase in the coronary death rate of smokers over nonsmokers (1). Positive correlation has also been established between the amount of smoking on one hand and both the earlier appearance of coronary occlusion and death from this cause (2). Laboratory studies utilizing rabbits have demonstrated that when nicotine is added to a hypercholesterolemic stimulus, significantly higher plasma cholesterol and lipid phosphorus levels result. The effect is essentially the same whether the cholesterol and nicotine are injected (3) or administered perorally (4). In both of these studies utilizing rabbits a minimal amount of cholesterol corresponding to approximately 0.1 per cent of the diet was utilized.

The purpose of this investigation was to determine whether the chicken is susceptible to the nicotine potentiation of hypercholesterolemia and whether a pathological differential may be established by the use of a normally atherogenic dietary level (1.0 per cent) of cholesterol (5).

EXPERIMENTAL AND DISCUSSION

White English Leghorn cockerels were raised on a diet of Purina Layena® pellets from two days to ten weeks of age. At this time four experimental groups of fourteen birds per group were established. Birds were randomly selected from a group of approximately equal weights. The experimental groups

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were as follows: (*a*), control; (*b*), nicotine (2.28 mg./Kg. per day in the drinking water); (*c*), cholesterol (1.0% in the feed); and (*d*), combined nicotine-cholesterol regimens. The daily dose of nicotine was roughly equivalent to the use of two packs of cigarettes by the human (4).

At ten weeks of age the birds were weighed and plasma cholesterol and phospholipid levels determined by methods previously described (4). Determinations were repeated at four-week intervals over the sixteen-week experimental period. At twenty-six weeks of age all birds were sacrificed and the hearts and aortas studied for pathological changes.

The control group gained weight at a slightly but not significantly greater rate than the other birds. Figure 1 is a record of the plasma total cholesterol levels and the cholesterol/phospholipid ratios over the sixteen-week period. The cholesterol and nicotine-cholesterol group values were not significantly different from one another because of rather large variations between birds. These variations were greatest at the eight and twelve-week periods when the differences in cholesterol levels and the ratios were most apparent. The effect of nicotine as a hypercholesterolemic or C/P stimulus is at best only suggestive.

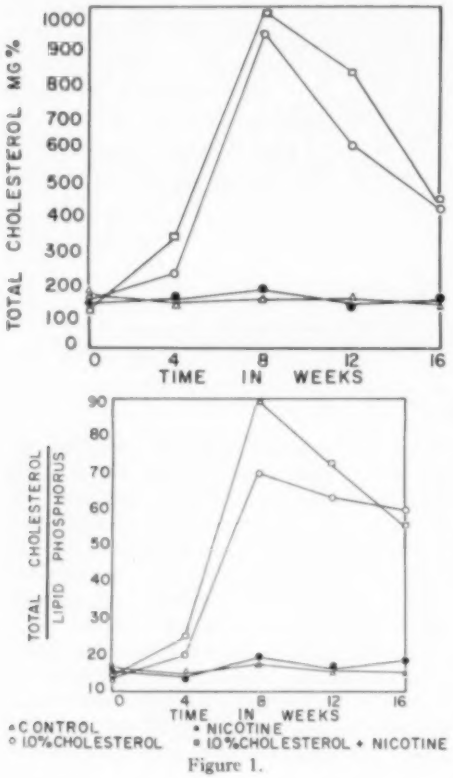

Figure 1.

The heart and entire aortas were removed from each animal. In each instance the aortas were grossly examined and five sections prepared from the tissues. The first included myocardium, leaflet of mitral valve, and ascending aorta with coronary arteries. The remaining sections consisted of a transverse section of the wall of the aorta, a transverse section of the inominate arteries, a transverse section of the left pulmonary artery, and an opened transverse section of the terminal aorta. In each instance H and E, toluidine blue, and von Kossa's stain were used. In addition, a separate block of the myocardium, aortic valve, coronary artery, and ascending aorta were studied by means of a frozen section with a fat stain.

The gross and microscopic studies of the control and nicotine groups revealed essentially no changes. Three birds of the cholesterol group demonstrated small yellow plaques in the aorta, none of which was greater than 2 mm. in diameter. Upon microscopic examination the plaques were found to consist of slightly thickened intima with a myxomatous, edematous-appearing fibrous tissue forming the main component. No other significant changes were noted in the cholesterol group.

Three birds of the nicotine-cholesterol group also demonstrated plaques in the aorta. Microscopic examination revealed an appreciable increase of fatty material in the thickened intima, particularly in the proximal aorta and aortic valve. There were occasional macrophages filled with a foamy material. The coronary vessels were essentially normal except for minimal thickening.

The fact that minimal pathologic changes observed in both cholesterol groups may indicate that either the particular strain of chicken employed possessed minimal atherogenic susceptibility or that spontaneous regression of the lesions had occurred. Evidence for the latter possibility may be the fall in cholesterol and C/P ratios at eighteen weeks of age. As earlier studies with the rabbit had established a marked effect of nicotine on blood cholesterol levels with a minimum (0.1%) diet of cholesterol, it is possible that the higher dose (1.0%) used in the study obscured any nicotine effect.

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The Analysis of Phenothiazine Derivatives with Tranquilizing Properties*

By JOHN MILNE

Nonaqueous assay methods have been developed for some of the more recently available phenothiazine derivatives with tranquilizing properties. These methods have been compared to Blazek and Stejskal's gravimetric procedure which has been successfully applied to these drugs in this laboratory as well as to the control procedures used by the pharmaceutical manufacturers. For purposes of nonaqueous titrimetry, the drugs studied may be divided into two groups by virtue of the monobasic or dibasic character of their N bonded side chain. The monobasic drugs are titrated in acetone and 2:1 hexane:acetone while the dibasic compounds which carry a piperazine ring in the side chain are titrated in 2:1 benzene:nitromethane. The differential titration of both the salts and bases of this last group in different solvent systems was studied before the benzene:nitromethane system was chosen and the results of this investigation are included.

With the advent of chlorpromazine and other similar phenothiazine derivatives with tranquilizing properties a need for analytical control procedures has arisen. Several methods have been introduced for these drugs and chemically similar antihistamines.

Colorimetry, involving both the reineckates (1) and the picrates (2), has been employed in the assay of these drugs. Brodie (3) complexed organic bases with acidic indicators and measured the colors formed, Porter and Silber (4) applied this technique to diethazine, and Haley and Bassin (5) utilized it for promethazine. Fossoul (6) used the color produced by the interaction of ammonium persulfate with promethazine, and Dubost and Pascal (7) determined chlorpromazine by measuring the color which it produces with concentrated sulfuric acid.

Ultraviolet spectrophotometry in water forms the basis of the United States Pharmacopeia procedure (8) for chlorpromazine tablets. This technique has also been applied to chlorpromazine, mepazine, and promethazine by Meyer (9) with ether and ethanol as solvents.

Several titration procedures have been reported among which are the argentometric titration (1) of the hydrochloride salts and nonaqueous titration of the sodium tetraphenylborates of both chlorpromazine and diethazine (1). Nonaqueous techniques have also been applied to promethazine hydrochloride by Kleckner and Osol (10), and to promazine and chlorpromazine by Milne and Chatten (11). Sandri (12) has determined promethazine, chlorpromazine, and diethazine by a bromination technique involving back titration of the excess bromide. The 1958 British Pharmacopoeia procedure for chlorpromazine is an acid-base back titration method (13) and the United States Pharmacopeia method for crystalline chlorpromazine is a nonaqueous titration procedure in glacial acetic acid (8).

Blazek (15) has simplified the original gravimetric method with silicotungstic acid which was introduced by Blazek and Stejskal (14). He has employed titration techniques with polarographic detection of the excess tungstate at the end point (15). Diethazine, chlorpromazine, and promethazine have been determined this way.

It was the purpose of this investigation to compare existing methods with the nonaqueous procedures developed in this laboratory, in an attempt to find suitable assays for these phenothiazine derivatives and their pharmaceutical preparations.

EXPERIMENTAL

Reagents.—Glacial acetic acid, A. C. S.; benzene, A. C. S.; chloroform, A. C. S.; acetone, A. C. S.; methanol, A. C. S.; ethanol, 95%; dioxane, Eastman Kodak; hexane, B. D. H. laboratory reagent; dimethylformamide, Anachemia, b. p. 152–154°; nitromethane, B. D. H. laboratory chemical or Eastman Kodak, practical grade, redistilled, b. p. 100–101°; 0.05 *N* perchloric acid in dioxane standardized against potassium acid phthalate, A. C. S., in glacial acetic acid with crystal violet as indicator; 0.05 *N* potassium hydroxide in methanol standardized against potassium acid phthalate, A. C. S., in 1:1 water-methanol with phenolphthalein as indicator; 0.1 *N* sodium methoxide in methanol standardized against benzoic acid, U. S. P., in dimethylformamide with thymol blue as indicator; 1% and 5% dimethyl yellow in chloroform; 1% tropaeolin 00 in methanol; 1% thymol blue in methanol; 6% mercuric acetate in glacial acetic

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The author wishes to express his appreciation for the helpful discussions with L. G. Chatten and his thanks to G. Morris for the figure.

acid; 10% silicotungstic acid solution; 40% potassium hydroxide solution; sodium sulfate, A. C. S.

Apparatus.—Fisher titrimeter, model No. 9-3111A, and Beckman pH meter, model G, with glass-indicator electrode and sleeve-type calomel reference electrode; 10-ml. semimicro-buret measuring to 0.02 ml.

Nonaqueous Procedures with Crystalline Materials[1]

Dibasic Drugs.—Prochlorperazine dimaleate (1-methyl-4-[3-(2-chloro-10-phenothiazinyl)-propyl]-piperazine dimaleate), thiopropazate dihydrochloride (1-(2-acetoxyethyl)-4-[3-(2-chloro-10-phenothiazinyl)-propyl]-piperazine dihydrochloride), Perphenazine (1-(2-hydroxyethyl)-4-[3-(2-chloro-10-phenothiazinyl)-propyl] piperazine).

An accurately known quantity (approx. 50 mg.) of the base is titrated in 2:1 benzene-nitromethane with total volume of about 150 ml. using perchloric acid in dioxane as titrant and 3 drops of tropaeolin 00 solution as indicator. The indicator color change is from yellow through amber to pink-violet or complete disappearance of the amber hue. Where the starting material is a salt the titration mixture may be obtained by extraction of an aqueous basic solution of the salt with six 15-ml. portions of benzene and subsequent addition of nitromethane. A minimum of potassium hydroxide solution should be employed while the aqueous solution should be nearly saturated with sodium sulfate in order to prevent emulsion.

The acid moiety of thiopropazate dihydrochloride may be titrated in chloroform (50-mg. crystalline material in 60-ml. solvent) with potassium hydroxide in methanol, or in dimethylformamide (150 mg. crystalline material in 50 ml. solvent) with potassium methoxide in methanol. Three drops of thymol blue indicator solution is used and the color changes are, respectively, yellow through green to full blue, and yellow to gray-green or complete disappearance of the yellow hue.

Monobasic Drugs.—Mepazine hydrochloride monohydrate (N-methylpiperidyl-(3)-methyl phenothiazine hydrochloride monohydrate), acepromazine maleate (10-(3-dimethylaminopropyl)-2-(acetyl) phenothiazine maleate), trifluprom azine hydrochloride (10-(3-dimethyl aminopropyl)-2-(trifluoromethyl)-phenothiazine hydrochloride).

An accurately known quantity of the salt (approx. 70 mg.) is dissolved in 50 ml. acetone and, in the case of hydrochloride salts, 0.5 ml. mercuric acetate solution is added. With mepazine and trifluprom azine, where 2 drops of 1% dimethyl yellow solution are added, the end point color change is from yellow to orange, whereas with acepromazine, where 2 drops of 5% dimethyl yellow solution are used, the end point color change is from orange-yellow to the first full orange color. Perchloric acid in dioxane is the titrant in all instances.

Mepazine hydrochloride is titrated as the base in 2:1 hexane-acetone by extraction of the salt in the same manner as that employed with the dibasic drugs but four 20-ml. portions of hexane are used and acetone is subsequently added. The procedure then follows exactly that used for the salt.

Gravimetric Procedures with Crystalline Materials

This procedure is applicable to all the above mentioned tranquilizing drugs except prochlorperazine dimaleate and follows the original technique of Blazek and Stejskal (14).

Accurately weigh approx. 0.06 M. E. (20 mg. with monobasic drugs and 10 mg. with dibasic drugs) of the compound into a beaker containing 20 ml. distilled water and 1 ml. concentrated hydrochloric acid, warm the solution to 70° and add, dropwise, 8 ml. silicotungstic acid solution. Collect the precipitate in a tared, sintered-glass crucible, washing with 50 ml. distilled water, and dry to constant weight.

Nonaqueous Procedures with Pharmaceutical Preparations

Prochlorperazine Dimaleate Tablets.—Weigh and powder 20 tablets. Transfer an accurately weighed quantity of the tablets equivalent to 50 mg. prochlorperazine into a 250-ml. separatory funnel containing 150 ml. distilled water, 0.2 ml. potassium hydroxide solution and approx. 3 Gm. sodium sulfate. Proceed as in crystalline prochlorperazine dimaleate method extracting with six 15-ml. portions benzene.

Suppositories.—Dissolve 20 suppositories in benzene contained in a 250-ml. volumetric flask and make up to the mark with the same solvent. Take an aliquot of 40 ml., add 20 ml. nitromethane, 1 drop tropaeolin 00 indicator solution, and titrate with perchloric acid in dioxane. The end point is the same as that observed with the crystalline prochlorperazine dimaleate determination.

Thiopropazate Dihydrochloride Tablets.—Proceed as directed for the prochlorperazine dimaleate tablets determination.

Mepazine Hydrochloride Monohydrate, Acepromazine Maleate, and Trifluprom azine Hydrochloride Tablets.—Weigh and powder 20 tablets. Accurately weigh a quantity of tablet mass equivalent to about 70 mg. of the drug and extract with 50 ml. of acetone for ten minutes, employing an electromagnetic stirrer. Filter through a fine sintered-glass funnel with the aid of suction, washing the beaker and residue with 30 ml. acetone. Proceed as directed in the determination of the crystalline materials for each particular compound beginning after the dissolution of the pure drug in acetone.

Mepazine Ampuls.—Empty the contents of 10 ampuls into a beaker and pipet a quantity of ampul solution equivalent to about 70 mg. mepazine hydrochloride into a 125-ml. separatory funnel containing 25 ml. distilled water, 0.2 ml. potassium hydroxide solution, and 1 Gm. sodium sulfate. Proceed as directed in the crystalline mepazine hydrochloride monohydrate determination as the base extracting with four 20-ml. portions of hexane.

Gravimetric Procedures with Pharmaceutical Preparations

Acepromazine, Perphenazine, and Trifluprom azine Tablets.—Weigh and powder 20 tablets. Weigh a quantity of the tablet mass equivalent to about 0.15 milliequivalents of active ingredient and

[1] In all nonaqueous procedures the blank for the particular solvent system used should be determined using the same indicator and end point color change as described in the procedures themselves.

extract with 25 ml. ethanol for ten minutes employing an electromagnetic stirrer. Filter through a fine sintered-glass funnel with the aid of suction, washing the beaker and residue with 30 ml. ethanol. Evaporate the ethanol and redissolve the residue in 10 ml. distilled water containing 0.5 ml. concentrated hydrochloric acid. Transfer to a 50-ml. volumetric flask with the aid of hydrochloric acid solution of the same concentration and make up to the mark with the same acid solution. Take a 20-ml. aliquot, filter if necessary, and proceed as directed for the determination of the crystalline material beginning at "warm the solution . . . ."

Acepromazine Ampul and Drop Solutions.—Take an aliquot equivalent to 0.06 M. E. of active ingredient from the solution of 10 ampuls or the drops solution, make up to 20 ml., and add 1 ml. concentrated hydrochloric acid. Proceed as directed for the determination of the crystalline material beginning at "Warm the solution . . . ."

DISCUSSION

The phenothiazine derivatives investigated in this work may be classified by their monobasic or dibasic character. The monobasic drugs trifluprom-azine hydrochloride, mepazine hydrochloride monohydrate, and acepromazine maleate were assayed similarly to the hydrochlorides of chlorpromazine and promazine as outlined in a previous publication from this laboratory (11). Dimethyl yellow indicator, however, was used in place of methyl red. Mercuric acetate was employed with the hydrochloride salts in both instances. Assay results and standard deviations for crystalline drugs are given in Table I and those for pharmaceutical forms in Table II.

For solubility reasons, extraction from aqueous basic medium was employed with prochlorperazine dimaleate and thiopropazate dihydrochloride while direct dissolution in the extraction solvent was used for perphenazine base. The water-immiscible extraction solvents chosen were benzene, chloroform, and hexane and for potentiometric titration purposes these were mixed with nitromethane, acetonitrile, and acetone. While differential titration curves were obtained in all instances except hexane:nitromethane and hexane:acetonitrile which were immiscible combinations, the second potential break was sharp enough only in the hexane:acetone and benzene:nitromethane systems to warrant further investigation. Only the latter system was used in this work, the hexane:acetone combination being the subject for future investigation. Tropaeolin 00, the indicator used by Yokoyama and Chatten (18) in the benzene:nitromethane:formic acid system was found to be satisfactory for the titration of these dibasic drugs.

Pifer and Wollish (17) have reported that the addition of an aprotic solvent to a polar solvent sharpens the end point. In the hexane:acetone and benzene:nitromethane combinations such addition appears to improve the second potential break but at the expense of the first end point in both instances. The separate components of the benzene:nitromethane system have been employed before for the titration of bases by various workers (19, 20, 21). However, Chatten, Pernarowski, and Levi (22) have reported a disadvantage with nitromethane. Apparently amines with pKb values less than 11 display two potential breaks when titrated in nitromethane. They have shown that the formation of a nitromethane addition product with the amine is possible and postulate that its formation accounts for the second potential break and limited success in the titration of amines reported by Fritz and Fulda (20). Although it has been observed here that acepromazine and mepazine display two potential breaks when titrated in benzene:nitromethane (2:1), no such phenomenon was encountered upon titration of the dibasic drugs in this system nor have Ciaccio, *et al.* (21), reported any trouble in their work with nitromethane. However, as an added precaution against erratic recoveries due to such interference, the second and last potential break was used for the calculation of recoveries. Tropaeolin 00 changes color at this potential break and the curve and color changes depicted in Fig. 1 are representative of the titrations of all the dibasic drugs in benzene:nitromethane.

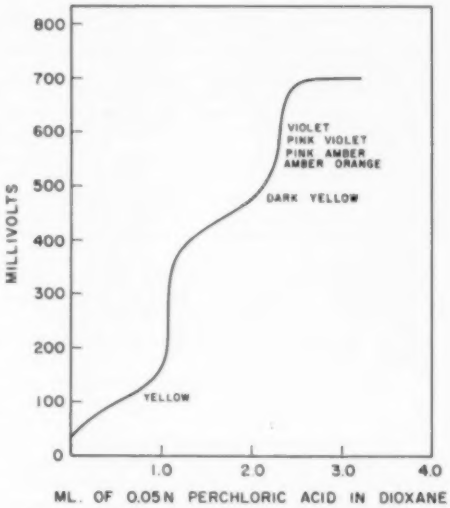

Fig. 1.—Titration of prochlorperazine base from crystalline prochlorperazine dimaleate in a 2:1 mixture of benzene:nitromethane using the Beckman pH meter.

Although the benzene:nitromethane system was chosen specifically for the assay of the pharmaceutical forms of the dibasic drugs and its application to crystalline drug was successful, its use was limited with the manufactured product. Prochlorperazine dimaleate and thiopropazate dihydrochloride were successfully determined in tablets but thiopropazate dihydrochloride gave erratic recoveries, indicated by a large standard deviation (see Table II). The recoveries by the procedure for perphenazine tablets were both low and erratic. Upon investigation of the tablet formulation, the activated charcoal, present as an excipient, was shown to be the cause. The results for all the crystalline dibasic drugs and their pharmaceutical forms along with standard deviations are given in Tables I and II, respectively.

TABLE I.—RESULTS OF THE ANALYSES OF CRYSTALLINE DRUGS

Crystalline Form of Drug	Mol. Wt.	Gravimetric Conversion Factor	Gravimetric Recovery, %	Nonaqueous Recovery, %	Nonaqueous Standard Deviation	Control Procedure Recovery, %
Trifluprom azine hydrochloride	388.88	0.3658	99.52	99.20	0.425	...
Acepromazine maleate	444.52	0.4267	99.68	100.06	0.144	100.6
Mepazine hydrochloride monohydrate	364.93	0.3574 0.3514 (with one mole water)	100.80 99.80	99.14 (as salt) 99.02 (as base)	0.245 0.446	...
Prochlorperazine dimaleate	606.07	...	...	98.96	0.253	100.4
Perphenazine	403.99	0.2213	98.50	99.50	0.364	...
Thiopropazate dihydrochloride	519.94	0.2847	98.11	98.06	0.447	98.98 (in $CHCl_3$) 98.05 (in DMF)

TABLE II.—RESULTS OF THE ANALYSES OF PHARMACEUTICAL FORMS

Pharmaceutical Form	Brand Name	State of Active Ingredient	Nonaqueous Recovery, %	Nonaqueous Standard Deviation	Gravimetric Recovery, %	Control Procedure Recovery, %
Trifluprom azine tablet	Vesprin	Hydrochloride	99.13	0.655	98.20	99.38
Acepromazine tablet	Plegicil	Maleate	97.01	0.590	97.84	108.00
Acepromazine drops	...	Maleate	98.36	...	103.2	111.00
Acepromazine ampul	...	Maleate	95.23	...	98.16	92.00
Mepazine tablet	Pacatal	Hydrochloride	100.18	0.610	...	98.96
Mepazine ampul	...	Acetate	102.10	0.150	...	99.04
Prochlorperazine tablet	Stemetil	Dimaleate	96.63	0.680	...	97.01
Prochlorperazine suppository	...	Free base	102.10	0.162	...	...
Perphenazine tablet	Trilafon	Free base	...	...	103.2	102.55
Thiopropazate tablet	Dartal	Dihydrochloride	99.67	1.62	...	98.24

Both the Fisher titrimeter and Beckman pH meter with glass-sleeve type calomel electrode combination were employed and perchloric acid in dioxane was the titrant in all instances. Where the titration solvent was water miscible, leakage of the salt bridge solution from the calomel electrode caused the potential and indicator color change to reverse. Replacement of the aqueous salt bridge with a potassium chloride saturated solution of the titration solvents gave erratic results and slow response. The silver:silver chloride reference electrode was tried but it afforded a confined potentiometric range less than one half the range offered by the calomel electrode and could only be used for the titration of the monobasic drugs. However, it was found that the application of grease to the sleeve of the calomel electrode prevented leakage and consequently allowed its use in all cases.

The Blazek and Stejskal gravimetric procedure was applied with success to all the crystalline compounds except prochlorperazine dimaleate. The structure of all silicotungstic acid precipitates was assumed to be four equivalents of nitrogen contributed by the drug to one equivalent of silicon contributed by the silicotungstic acid. Table I shows the value of the gravimetric conversion factor used for each precipitate for conversion to the quantity of crystalline drug originally present in the form used, along with percentage recoveries of pure drug calculated by use of these constants. With the constant for thiopropazate it was assumed that the drug hydrolyses completely to perphenazine, proof of which is indicated by comparison of the recoveries using this constant with those arrived at by other methods. Two constants and percentage recoveries are given for mepazine, the first assuming the precipitate to contain one mole of water as does the hydrochloride salt, the second assuming the absence of water. By comparison with nonaqueous results, the first constant appears to give recoveries closer to the correct figure. This gravimetric procedure has been applied to the following pharmaceutical preparations; trifluprom azine tablets, acepromazine tablets, ampuls and drops, and perphenazine tablets. The results appear in Table II.

The control methods which were developed by the pharmaceutical manufacturers for their products were also investigated and compared to the other procedures. In all instances but one, prochlorperazine suppositories, at least two methods of

assay, selected from nonaqueous, gravimetric, and control procedures were performed in order to verify results.

Two control procedures have been developed by the manufacturers of prochlorperazine (23) for the determination of crystalline prochlorperazine dimaleate and its pharmaceutical preparations; (*a*) nonaqueous titration of the acid moiety (maleic acid) in dimethylformamide with sodium methoxide in methanol as titrant and thymol blue as indicator and (*b*) nonaqueous titration of the drug in glacial acetic acid with perchloric acid as titrant and α-naphthol benzein as indicator. Neither with the pure substance nor the pharmaceutical forms was it possible in this laboratory to obtain a sharp end point in the former procedure and the accuracy of the method was doubted for this reason. However, results submitted by the manufacturer and those obtained with the same lot of tablets by the nonaqueous method developed in this laboratory compared favorably (see Table II). The titration in glacial acetic acid was limited to pure drug only and the results are given in Table I.

In order to verify the results of the nonaqueous procedure developed here for thiopropazate dihydrochloride using the benzene:nitromethane solvent system, two additional nonaqueous procedures were developed and used, both of which involve the titration of the acid moiety (hydrochloric acid) of thiopropazate dihydrochloride. In the first instance, the drug is titrated in dimethylformamide with sodium methoxide in methanol while, in the second, the solvent is chloroform and the titrant, potassium hydroxide in methanol. When the potentiometric titration curves were taken, differential titrations were observed and the indicator, thymol blue, in both instances changed at the second millivolt maximum. The chloroform curve could not be detected initially because the meter readings were very unsteady until sufficient methanol from the titrant had been added. Table I gives the recoveries by both methods.

An attempt was made to apply both of these procedures to thiopropazate tablets but with little success. The dimethylformamide titration did not give a sharp indicator color change or potentiometric break and the chloroform extraction and titration gave low results. Tablet formulations were made up and certain excipients eliminated in order to determine the cause of the low recoveries in the chloroform titration and it was found that the sodium sulfate, which was present in large quantity, was the interfering ingredient. Since no acidic excipients were present in the tablet formulation, it was felt that extraction and titration in chloroform:methanol might be possible. However, low recoveries were still obtained with the tablets and when crystalline product was titrated, the second potential break and indicator color change deteriorated with the increased proportion of methanol. In the control procedure submitted by the company (24), the compound is determined by the potentiometric titration of the ionic chloride with silver nitrate using the glass-silver electrode combination. The results of this procedure with tablets are given in Table II.

Titration of crystalline thiopropazate dihydrochloride as the acetate in chloroform:nitromethane was also tried. Although no sharp potential breaks resulted and thus the method could not be used, it is interesting to note that a differential titration was obtained. Ciaccio, *et al.* (21), have explained the differential titration of the free bases of piperazine derivatives by the decreased strength of the second nitrogen after neutralization of the first. They explain that the presence of a proton on the neutralized nitrogen lessens the attraction of a proton to the remaining nitrogen. The explanation may be used to account for the differential titration of the acetate in chloroform:nitromethane. The differential acid moiety titrations in dimethylformamide and chloroform may be accounted for similarly by the enhanced proton affinity of the basic nitrogen carrying the untitrated hydrochloride group after removal (by neutralization) of the hydrochloric acid from the other nitrogen. Ciaccio, *et al.* (21), noted that disubstituted piperazine bases displayed only one potentiometric break in glacial acetic acid when titrated with perchloric acid in acetic acid. It has been found that thiopropazate acetate, formed from the hydrochloride with mercuric acetate, acts similarly when titrated with perchloric acid in dioxane. Because perchloric acid is sufficiently strong to partially titrate thiopropazate dihydrochloride, an attempt to titrate the basic excipients before addition of mercuric acetate was unsuccessful.

The control procedure for perphenazine is an ultraviolet spectrophotometric procedure with methanol as solvent (25). Comparisons with a standard solution are read at about 257 mμ. Mepazine is determined similarly with ethanol as solvent for tablets (water for ampuls) and readings are taken at about 254 mμ (26). The results of these procedures as applied to commercial preparations are given in Table II.

For control purposes crystalline acepromazine maleate was determined in glacial acetic acid with perchloric acid as titrant and crystal violet as indicator (27). The results of this assay are given in Table I. However, the commercial forms of this drug were determined by a colorimetric procedure using a sulfuric acid-bromine water reagent (27). Erratic results were obtained by this method even within the readings of the standard solutions. The results of this technique are given in Table II also. Because of the erratic nature of all the readings taken in this procedure and the low recoveries obtained by nonaqueous titrimetry for the aqueous preparations of both pure drug and commercial products, it was felt that the gravimetric recoveries were the most accurate and are the accepted results for the aqueous preparations. The reliability of the gravimetric procedure is also borne out by the fact that the gravimetric and nonaqueous results agree well for both the tablets and crystalline drug.

The control method for triflupromazine tablets is also an ultraviolet spectrophotometric procedure (28) similar to that given for chlorpromazine in the U. S. P. XV supplement (8). Results of this procedure are also given in Table II.

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Some Aspects of the Central Nervous System Activity of a Series of Urazole Derivatives*

By C. L. MITCHELL,† H. H. KEASLING, and E. G. GROSS

The neurotoxicity, antielectroshock, anti-Metrazol, and antistrychnine activities of a series of urazole compounds have been determined in mice. The compounds were relatively short acting and nontoxic. In general, the members of the series were found to be highly specific against maximal Metrazol seizures. Variations in the substituted nitrogen groups yield compounds possessing anti-Metrazol, antistrychnine, convulsant, and depressant type actions.

THE SIMILARITY of the urazole and hydantoin nucleus prompted the present investigation of the C. N. S. activity of a series of substituted urazoles. The structures of the derviatives studied are detailed in Table I. The compounds were administered to mice and their observable effects were noted. In addition, their antielectroshock, anti-Metrazol, and antistrychnine activities were tested. This report details the results of these experiments and some observations with regard to the structure-activity-relationship of their C. N. S. actions.

EXPERIMENTAL

Adult male albino mice obtained from the Carworth Farms (CF No. 1 strain) were maintained on Purina laboratory chow and allowed free access to food and water except during the test period. The mice were used for one experiment only. The test procedures employed were: (*a*) the supramaximal electroshock seizure technique, M. E. S., of Toman, *et al.* (1), in which alternating current, 50 ma. of 0.2 second duration was delivered through Spiegel corneal electrodes (2) from an electroshock apparatus constructed according to the design of Woodbury and Davenport (3); (*b*) the maximal Metrazol seizure test, M. M. S. of Goodman, *et al.* (4), in which Metrazol,[1] 38 mg./Kg., was injected intravenously. The end points for both tests were the abolition of the tonic extensor components of the seizure pattern; and (*c*) ability to prevent death from strychnine, in which 2.5 mg./Kg. of strychnine was injected intraperitoneally (5).

All compounds were suspended in 10% gum acacia for oral administration. The convulsant stimulus was originally delivered one hour following urazole administration but early in the testing at 1 Gm./Kg. it was noted that the compounds were rapidly absorbed and of short duration. Therefore, subsequent tests at 1 Gm./Kg. and all lower doses were determined thirty minutes after dosage. Phenobarbital, trimethadione,[2] and diphenylhydantoin[3] were tested at the optimum times suggested by Swinyard, *et al.* (6). Those compounds exhibiting protection at 1 Gm./Kg. from electroshock and/or Metrazol were then administered in dosages of 300, 100, and 30 mg./Kg. until less than 50% of the animals were protected.

Acute neurotoxicity (NT) was determined by the method of Swinyard, *et al.* (6). The following end points were used: (*a*) positional sense test, (*b*) righting test, (*c*) gait and stance test, (*d*) muscle tone test, and (*e*) equilibrium test. Neurotoxicity was recorded when an alteration in response to the above procedures was observed. The mice were examined at fifteen minute intervals for at least one hour and thereafter until two successive examinations indicated no change, or until a decrease in the number of mice exhibiting neurological symptoms was seen.

The 50% neurotoxic dose (TD_{50}) and the 50%

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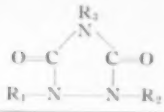
† Smith, Kline and French Fellow in Pharmacology. The material contained in this report was submitted to the Graduate College of the State University of Iowa in partial fulfillment of the requirements for the degree of M.S. The urazole compounds were kindly supplied by, and the work was supported in part by, the Knoll Pharmaceutical Co., Orange, N. J.

[1] The Metrazol was kindly supplied through the courtesy of Dr. R. O. Hauck, Knoll Pharmaceutical Co.
[2] The trimethadione was kindly supplied through the courtesy of Dr. R. K. Richards, Abbott Laboratories.
[3] The diphenylhydantoin was kindly supplied through the courtesy of Dr. Graham Chen, Parke, Davis and Co.

TABLE I.—STRUCTURES OF URAZOLE DERIVATIVES



UR No.	R_1	R_2	R_3
4	H	H	H
5	H	H	CH_3
7	H	CH_3	H
37	H	H	NH_2
1	H	ø	H
3	H	ø	CH_3
8	CH_3	ø	H
9	CH_3	ø	CH_3
46	CH_3	ø	CH_2CH_2OH
47	CH_3	ø	n-C_3
10	n-C_3	ø	H
12	iso-C_3	ø	H
23	n-C_3	ø	H
25	C_2	ø	H
26	n-C_3	ø	n-C_3
27	iso-C_3	ø	n-C_3
28	iso-C_4	ø	H
49	iso-C_5	ø	H
50	iso-C_5	ø	CH_3
51	iso-C_5	ø	n-C_3
52	iso-C_5	ø	iso-C_3
53	iso-C_5	ø	CH_2CH_2OH
34	iso-C_5	iso-C_3	H
35	iso-C_5	iso-C_3	iso-C_3
36	iso-C_5	iso-C_3	n-C_3
38		ø	

UR No.	R_1	R_2	R_3
40		ø	H
17		H	
20		ø	H
24			n-C_3
29		ø	n-C_3
42		ø	CH_3
44		ø	CH_2CH_2OH
15	$CH_2ø$	ø	H
30	$CH_2ø$	ø	n-C_3
31	$CH_2ø$	ø	$CH_2ø$
43	$CH_2ø$	ø	CH_3
45	$CH_2ø$	ø	CH_2CH_2OH
2	ø	ø	H
6	ø	ø	CH_3
11	ø	ø	iso-C_5
13	ø	ø	n-C_3
14	ø	ø	ø
16	ø	ø	CH_2CHCH_2
18	ø	ø	CH_2CH_2OH
19	ø	ø	CH_2CH_2Cl
21	ø	ø	C_2
22	ø	ø	n-C_5
32	ø	ø	
33	ø	ø	
39	ø	ø	
41	ø	ø	

effective dose (ED_{50}) of the urazole compounds were determined thirty minutes after the administration of the drug. Similar tests for trimethadione, phenobarbital, and diphenylhydantoin were determined at one and one-half hours, two hours, and three hours, respectively. The TD_{50} or ED_{50} was obtained by plotting the effect of at least 3 dose levels yielding between 10 and 90% effect on log-probit graph paper and fitting the points with a straight line. At least 10 mice were utilized in determining each point. The computations for fitting the probit-log dose regression line, the determination of the TD_{50}, ED_{50}, protective index (P. I.), ratio of ED_{50} M. E. S./ED_{50} M. M. S., and the 95% confidence interval were done according to the method of Finney (7).

The time of peak anticonvulsant effect and duration of action of one compound (UR 20) was determined by the M. M. S. test. An approximate ED_{60} of UR 20 was administered to 6 groups of 10 animals. The respective groups of mice were then injected with Metrazol at fifteen, thirty, forty-five, sixty, ninety, and one hundred and twenty minutes.

The sleeping time produced by two compounds (UR Nos. 12 and 23) was compared with hexobarbital. Sleeping time was recorded as the time interval in which the righting reflex of the mice was absent. The effect of two convulsant compounds (Nos. 35 and 36) upon the sleeping time of hexobarbital was also determined. The convulsant compounds were administered orally, two minutes prior to the i. p. injection of hexobarbital. The 95% confidence intervals were calculated according to the method of Snedecor (8).

RESULTS

In general, the compounds were relatively nontoxic. No mice were killed at 1 Gm./Kg. with the exception of UR Nos. 34, 35, and 36 which were lethal convulsants (*vide infra*), UR Nos. 12 and 23 which produced stimulation followed by profound depression (*vide infra*), and UR No. 10. The predominant neurotoxic effect was ataxia. Flaccid paralysis was noted following administration of UR 8.

The anticonvulsant activities are detailed in Table II. Our data for phenobarbital (TD_{50}, 60 mg./Kg., ED_{50} M. E. S., 21 mg./Kg.) and diphenylhydantoin

TABLE II.—ANTICONVULSANT ACTIVITY OF URAZOLES[a, b, c]

UR No.	Test	1,000	300	100	30	UR No.	1,000	300	100	30
8	NT[d]	7/20	0/10	..	..	..	11/20	3/10	0/10	..
8	MES[e]	7/10	4/10	..	..	20	8/10	7/10	0/10	..
8	MMS[f]	5/5	5/5	0/10	..	..	5/6	8/10	6/10	3/10
9	NT	4/10	0/10	..	..	..	0/20	0/10	..	..
9	MES	9/10	0/10	..	..	42	0/10	..	..	..
9	MMS	5/5	0/10	..	..	..	7/10	0/8	..	..
46	NT	2/20	0/20	0/10	..	..	1/20	0/10	..	..
46	MES	10/10	0/10	..	..	44	0/10	..	..	..
46	MMS	10/10	4/7	1/8	..	..	6/10	2/10	..	..
47	NT	6/20	0/20	..	..	..	0/20	0/10	..	..
47	MES	6/10	0/10	..	..	30	5/9	0/10	..	..
47	MMS	10/10	3/9	..	..	..	5/8	0/10	..	..
10	NT	20/20	9/10	1/10	..	..	0/20	0/10	..	..
10	MES	10/10	10/10	2/10	..	43	0/10	..	..	..
10	MMS	5/5	10/10	10/10	2/10	..	5/8	0/10	..	..
25	NT	20/20	5/10	0/10	..	..	0/20	0/10	0/10	..
25	MES	10/10	7/10	0/10	..	45	5/10	0/10	..	..
25	MMS	5/5	10/10	8/8	2/10	..	10/10	5/8	0/10	..
26	NT	7/10	2/10	0/10	..	..	20/20	5/10	0/10	..
26	MES	10/10	7/10	0/10	..	2	10/10	7/10	1/10	..
26	MMS	4/5	9/10	3/10	..	..	5/5	10/10	7/10	1/10
28	NT	20/20	6/10	0/10	..	..	4/10	0/10	..	..
28	MES	10/10	9/10	0/10	..	6	7/10	0/10	..	..
28	MMS	5/5	9/10	4/10	..	..	5/5	4/10	..	..
49	NT	20/20	20/20	6/20	..	..	0/20	..	..	..
49	MES	10/10	10/10	0/10	..	16	3/10	0/10	..	..
49	MMS	8/8	9/9	8/8	2/8	..	7/8	4/10	..	..
50	NT	20/20	15/20	2/20	..	..	6/20	0/10	..	..
50	MES	7/10	7/10	0/10	..	18	9/10	0/10	..	..
50	MMS	7/8	6/6	3/4	1/8	..	4/8	0/10	..	..
51	NT	7/20	0/20	..	..	..	4/10	0/10	..	..
51	MES	5/10	0/10	..	..	32	10/10	0/10	..	..
51	MMS	8/8	1/10	..	..	..	5/5	6/9	0/10	..
53	NT	20/20	0/20	..	..	..	0/20	0/10	..	..
53	MES	5/10	0/10	..	..	39	0/10	..	..	..
53	MMS	10/10	0/10	..	..	..	4/5	6/9	3/8	..
40	NT	20/20	0/10	..	..	..	0/20	..	..	..
40	MES	8/10	0/10	..	..	41	0/10	..	..	..
40	MMS	5/10	3/8	..	..	..	3/8	..	..	..

[a] UR Nos. 4, 5, 7, 37, 1, 27, 52, 38, 17, 24, 29, 31, 11, 13, 14, 19, 21, 22, and 33 inactive at 1 Gm./Kg.
[b] UR Nos. 3, 34, 35, and 36 are convulsant (see text).
[c] UR Nos. 12, 15, and 23 stimulate, then depress at 1 Gm./Kg. (see text).
[d] NT = Neurotoxicity (see text).
[e] MES = Supramaximal electroshock technique (see text).
[f] MMS = Maximal Metrazol seizure test (see text).

(TD_{50}, 100 mg./Kg., ED_{50} M. E. S., 14 mg./Kg.) compare favorably with those of Swinyard, *et al.* (6), (phenobarbital—TD_{50}, 60 mg./Kg.; ED_{50} M. E. S., 20 mg./Kg., and diphenylhydantoin—TD_{50}, 90 mg./Kg.; ED_{50} M. E. S., 10 mg./Kg.). While the urazole compounds were relatively ineffective against M. E. S. in non-neurotoxic doses, good anti-M. M. S. activity was demonstrated.

The protective index against M. M. S. was determined for the more active members of the series. The compounds selected were UR-2, 10, 20, 25, and 49. The protective indexes of trimethadione and phenobarbital were also determined. These data are presented in Table III. Our data for the oral ED_{50} values of trimethadione and phenobarbital are in general agreement with the subcutaneous ED_{50} values of 198 mg./Kg. for trimethadione and 9 mg./Kg. for phenobarbital sodium, obtained by Goodman, *et al.* (4). The ratio of ED_{50} M. E. S./ED_{50} M. M. S. was determined for the above compounds and diphenylhydantoin. These data are presented in Table IV. The relative selectivity of UR 2, 20, and 49 as M. M. S. antagonists is particularly striking. Our data for the ratio of ED_{50} M. E. S./ED_{50} M. M. S. for phenobarbital sodium and trimethadione are in general agreement with Goodman, *et al.* (4), and Chen, *et al.* (9). Our ratio for diphenylhydantoin lies between the ratio of 0.19 obtained by Goodman, *et al.*, and the ratio of 2.1 obtained by Chen, *et al.* In the study of Goodman, *et al.* (4), the compounds were given subcutaneously, whereas we administered the compounds orally. Chen, *et al.* (9), used a modification of the intravenous Metrazol infusion technique, and an electroshock stimulus of different intensity than that used by us. These differences may account for the discrepancies obtained in the three laboratories.

Time-duration studies on UR 20, 100 mg./Kg. (Fig. 1), indicate that this compound has a peak effect fifteen minutes after administration. It possesses little, if any, protective action one hour after administration. The neurotoxicity tests indicated that all active members of this series are rapidly absorbed from the intestinal tract and have a relatively short duration of action.

UR 3 produced hyperactivity at a dose of 1 Gm./Kg. Only 3 out of 10 mice survived the electroshock stimulation, whereas we have found that control mice usually survive this test.

Compounds UR 34, 35, and 36 produced con-

TABLE III.—TOXICITY, ANTI-M. M. S. POTENCY, AND PROTECTIVE INDEXES OF UR 2, 10, 20, 25, 49, TRIMETHADIONE, AND PHENOBARBITAL[a]

Drug	Time,[b] min.	Neurotoxicity, TD_{50}, mg./Kg.	Potency, ED_{50}, mg./Kg.	Protective Index, P. I.
UR 2	30	295 (163–380)	70 (48–100)	4.2 (2.6–6.2)
UR 10	30	168 (125–221)	48 (34–60)	3.5 (2.5–5.3)
UR 20	30	452 (322–634)	73 (52–90)	6.6 (4.4–10.4)
UR 25	30	294 (224–385)	54 (37–70)	5.4 (3.8–7.9)
UR 49	30	151 (99–195)	38 (26–47)	4.2 (2.9–6.0)
Trimethadione	90	975 (760–1390)	236 (196–281)	4.0 (3.0–5.5)
Phenobarbital sodium	120	60 (47–77)	9 (6–12)	6.9 (4.8–10.1)

[a] Values in parentheses represent the 95% confidence interval.
[b] Time elapsed after administration of drug before Metrazol was injected.

TABLE IV.—ANTI-M. M. S. POTENCY, ANTI-M. E. S. POTENCY, AND RATIO ED_{50} M. E. S./ED_{50} M. M. S. FOR UR 2, 10, 20, 25, 49, TRIMETHADIONE, PHENOBARBITAL, AND DIPHENYLHYDANTOIN[a]

Drug	Time,[b] min.	M.M.S. Potency ED_{50}, mg./Kg.	M.E.S. Potency ED_{50}, mg./Kg.	Ratio ED_{50} M. E. S./ ED_{50} M. M. S.
UR 2	30	70 (48–100)	508 (404–619)	7.4 (5.1–10.4)
UR 10	30	48 (34–60)	136 (100–174)	2.9 (1.7–3.3)
UR 20	30	73 (52–90)	438 (359–528)	6.0 (4.6–8.1)
UR 25	30	54 (37–70)	262 (111–355)	4.8 (3.3–7.2)
UR 49	30	38 (26–47)	201 (133–271)	5.6 (3.9–8.5)
Trimethadione	90	236 (196–281)	1082 (923–1262)	4.6 (3.8–5.7)
Phenobarbital sodium	120	9 (6–12)	21 (17–25)	2.4 (1.7–3.3)
Diphenylhydantoin	180	11 (9–12)	14 (11–17)	1.3 (1.1–1.7)

[a] Values in parentheses represent the 95% confidence interval.
[b] Time elapsed after administration of drug before stimulus was delivered.

vulsions at 1 Gm./Kg. The mice receiving compound 34 exhibited only clonic seizures and died of respiratory failure within one hour. A dose of 500 mg./Kg. produced clonus within ten minutes. Seven out of 10 animals died within two hours. Compound 35, 1 Gm./Kg., produced death in 6 out of 10 mice within thirty minutes, while the remaining animals showed mild clonus and tonic flexor seizures for an hour. At 500 mg./Kg., all had periodic mild clonus and appeared stimulated for approximately forty-five minutes. UR 36, 500 mg./Kg., produced clonus which rapidly changed to tonic flexor and tonic extensor seizures. Four out of five mice died within ten minutes after dosage. Neither UR 35 nor UR 36, at doses of 100 mg./Kg. and 300 mg./Kg. had any apparent effect on the sleeping time of mice receiving 100 mg./Kg. of hexobarbital administered i. p. (Table V).

Compounds UR 12, 15, and 23, 1 Gm./Kg., caused initial stimulation which was followed by profound depression. Table VI shows the mean sleeping time of UR 12 and UR 23, 300 mg./Kg., as compared to a similar dose of hexobarbital.

All active compounds were studied for their ability to protect against death produced by i. p. injections of 2.5 mg./Kg. of strychnine. Only UR 8, 1 Gm./Kg., provided significant protection and was comparable to 250 mg./Kg. of Flexin[4] and 500 mg./Kg. of mephenesin (Table VII).

DISCUSSION

The qualitative changes in biological activity induced by variations in the substituent groups on the urazole nucleus provokes many speculations as to the nature of these relationships. In the following paragraphs certain of these relationships are detailed. However, the possibility exists that these speculations are not very reliable. The compounds designated as inactive produced no observable effects during a period of ninety minutes following oral administration of a dose of 1 Gm./Kg. At least three possibilities are pertinent to the present discussion: (*a*) These derivatives are delayed in onset beyond the period of observation, (*b*) they are not absorbed following oral administration, or (*c*) they are unable to alter the tested phenomena. The rapid onset and short duration of action of the active urazoles studied seems to render (*a*) unlikely. These

[4] The Flexin was kindly supplied through the courtesy of Dr. Charles F. Kade, Jr., McNeil Laboratories, Inc.

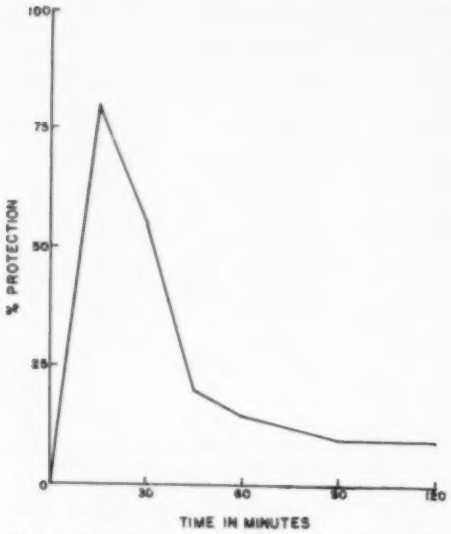


Fig. 1.—Duration of action of UR 20, 100 mg./Kg.

TABLE V.—EFFECT OF UR 35 AND UR 36 UPON THE MEAN SLEEPING TIME OF HEXOBARBITAL, 100 MG./KG. I.P. IN THE MOUSE

Hexobarbital, 100 mg./Kg. i. p. with:	Mean Sleeping Time, min.	95% Confidence Interval
Nothing	21	13.9–28.1
UR 35 100 mg./Kg. (oral)	24	11.8–36.2
UR 35 300 mg./Kg. (oral)	30	18.2–41.8
UR 36 100 mg./Kg. (oral)	29	19.5–38.5
UR 36 300 mg./Kg. (oral)	23	12.6–33.4

TABLE VI.—MEAN SLEEPING TIME OF UR 12, UR 23, AND HEXOBARBITAL, 300 MG./KG., ORALLY

Drug	Mean Sleeping Time, min.	95% Confidence Interval
UR 12	47	24.6– 69.9
UR 23	94	76.5–112.6
Hexobarbital	59	40.0– 77.8

TABLE VII.—ABILITY TO PREVENT DEATH FROM I. P. INJECTION OF 2.5 MG./KG. OF STRYCHNINE

Drug	Oral Dose	Time,[a] min.	No. of Animals Surviving/ No. of Animals Tested
UR 8	1 Gm./Kg.	30	4/8
Flexin	250 mg./Kg.	60	4/10
Mephenesin	500 mg./Kg.	5	4/10

[a] Time elapsed after administration of drug before strychnine was injected.

same considerations apply to (*b*) and, in addition, the presence of activity in closely related derivatives would seem additional evidence negating the validity of (*b*). We have therefore assumed that (*c*) is in fact true, and upon this hypothesis we have based the following discussion of structure-activity-relationships.

The results obtained from this series indicates that at least one phenyl group on the adjacent nitrogen atoms (R_1 or R_2, Table I) is essential for anticonvulsant activity. The urazole nucleus alone (UR 4) is devoid of anti-MES and anti-MMS activity. Note also UR 4, 7, and 37. Cyclohexyl substituents on both the R_1 and R_2 positions (UR 17) are also inactive, whereas a cyclohexyl substituent on one symmetric nitrogen with a phenyl group on the other (UR 20) was one of the more active compounds tested. Isopropyl substituents on both R_1 and R_2 (UR 34, 35, and 36) confer convulsant properties.

If R_1 or R_2 is phenyl, all other substituents except hydrogen on the adjacent nitrogen atom can confer anticonvulsant activity. (Compare UR 1 and UR 3 with 8, 9, 10, 12, 23, 25, 28, 40, 20, 12, 2, and 49). It is interesting to note that UR 3 and UR 8 have similar structures except for a reversal of the methyl group from the R_3 position (UR 3) to one of the adjacent nitrogen atoms (UR 8) resulting in a change of activity from stimulatory (UR 3) to anticonvulsant (UR 8).

The R_3 substituent must be small or relatively polar for maximal anticonvulsant activity. A hydrogen atom on the R_3 position possesses the greatest activity. Substitution of a methyl group for the hydrogen atom results in a reduction in potency. Any group larger than a methyl group, unless it is somewhat polar, produces little, if any, anti-MES or anti-MMS activity. (Compare the series UR 8, 9, 46, and 47; UR 10 and 26; UR 12 and 17; UR 20, 42, 44, and 29; UR 15, 45, 43, 30, and 31; UR 2, 6, 16, 18, 39, 11, 13, 14, 19, 21, 22, 32, and 33; UR 49, 50, 53, 51, and 52). The only example in this series of a completely polar group (UR 41) was found to possess little activity.

It is difficult to formulate structure-activity-relationships for convulsant, antistrychnine, or hexobarbital-like activity in view of the few compounds possessing these actions.

The variety of actions noted in this series is certainly of interest, although the anti-MMS activity is perhaps the most striking property of the urazoles

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Gibberellic Acid: Toxicologic and Pharmacologic Studies*

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The plant growth stimulant, gibberellic acid, was tested for its toxicologic and pharmacologic properties in mice, rats, cats, guinea pigs, rabbits, and dogs. It was essentially nontoxic when tested by various routes of injection in mice for its acute toxicity. Both subacute toxicity in mice, and subchronic toxicity in dogs and rats showed the compound to be asymptomatic and free of pathologic changes, histologically. The following studies were likewise negative: circulation and respiration, barbiturate potentiation, antipyresis, anticonvulsant activity, intestinal motility, blood cholesterol, and blood glucose.

In 1932, Kurosawa (1) reported that culture filtrates of the fungus *Gibberella fujikuroi* produced a substance which caused an overelongation phenomenon of rice plants. Within the last several years, interest has centered on the practical application of this plant growth-promoting substance called the gibberellins or gibberellic acids in the fields of horticulture and agriculture. Though voluminous reports have appeared on the effects of this substance in plants, relatively few have appeared concerning its possible effects on mammalian organ systems. It was, therefore, considered of interest to report on our toxicologic and pharmacologic studies with this plant stimulant. The results obtained provide the basis for this report.

EXPERIMENTAL

Material.—The sodium salt of gibberellic acid[1] was used as an aqueous solution in concentrations up to 30% for intravenous toxicity studies. For mouse studies, the volume injected intravenously was kept below 0.5 ml. and the speed of injection at 0.5 ml./minute. Methylcellulose suspensions of the drug were used for oral toxicity studies. For subchronic toxicity studies in rats, aqueous solutions of the drug were freshly prepared every 3–4 days and kept under refrigeration when not in use. For all other parenteral studies, drug solutions were prepared on the day of the experiment.

Acute Toxicity in Mice.—The drug was tested for its acute oral, intravenous, and intraperitoneal toxicity in groups of adult male Rockland mice, weighing between 18–24 Gm., using 10 animals per dose level. Animals were observed for two weeks following medication.

Subacute Toxicity in Mice, Dogs, and Rats.—Ten adult Rockland mice were medicated subcutaneously for fourteen days at 1 Gm./Kg., and 5 mice were dosed intravenously for five days at 2 Gm./Kg. To minimize the possibility of irritation due to repeated trauma of subcutaneous injection, four different areas were selected and used in sequential order during medication. Saline controls were run concurrently. Animals not sacrificed for autopsy were observed for an additional nine days.

Two litter-mate dogs of both sexes were medicated orally via capsule with 1 Gm./Kg. of the drug, six days a week for a period of ninety days. Six male and six female Holtzman rats were treated orally with a solution of the drug via feeding tube at the same dose level for a similar period. The following laboratory procedures were performed: urinalysis (clinical and microscopic), complete blood counts (hemoglobin, red, white, and differential), function tests (liver and kidney), and blood chemistry (glucose, nonprotein nitrogen, and bilirubin).

Circulatory and Respiratory Studies.—Dogs and cats, anesthetized with Nembutal sodium, were used to record circulatory changes from the carotid. Intrathoracic and intrapulmonic fluctuations in dogs were recorded with tambours via Jackson-spike and tracheal cannula. Drug injections were made into the femoral vein.

C.N.S. Effects.—The effect of the gibberellins on barbiturate potentiation was tested in groups of 10 male albino mice, pretreated either intravenously or orally with the drug at dose levels of 500 and 1,000 mg./Kg., respectively. Following pretreatment periods of ten minutes for the intravenous route, and forty-five minutes for the oral route, the animals were injected intravenously with 30 mg./Kg. of Pentothal sodium.

Anticonvulsant activity was evaluated by the use of a battery of three tests as previously outlined by Toman, Everett, and Richards (2). It consisted of the Metrazol, "psychomotor" and supramaximal electroshock tests.

Antipyretic activity was measured by taking normal temperatures hourly over a three-hour period in groups of four female Holtzman rats, followed by an initial intramuscular injection of 1.5 ml. of 15% Brewer's yeast. Eighteen hours later, the animals were challenged intramuscularly with a 1 ml. pyrogenic booster injection of yeast suspension. At this point the animals were medicated orally with the gibberellins at dose levels of 150 and 500 mg./Kg. and its possible antipyretic effects tested at one-half hour intervals for a period of two and one-half hours.

In Vitro Intestinal Motility.—The effect of the drug against spontaneous intestinal motility was tested on the rabbit ileum, using the Magnus method. The effects of the drug against standard spasmogens and relaxants (acetylcholine, histamine, barium, pilocarpine, epinephrine, and atropine) were carried out with guinea pig and rabbit ileum.

* Received July 21, 1958, from Department of Pharmacology, Research Division, Abbott Laboratories, North Chicago, Ill.

[1] Gibberellic acid, Abbott (Peoria Strain, 91% X and 9% A).

Sixteen rabbits and four guinea pigs were used in these studies.

Other Studies.—Total blood plasma cholesterol determinations were made in groups of six mice given the gibberellic acid in their diet at a level of 0.17%, equivalent to about 249 mg./Kg. daily for two weeks. The method of Zak, *et al.* (3), was used to determine total cholesterol.

Blood glucose levels were determined four hours after oral doses of 250 mg./Kg. and 500 mg./Kg. of the gibberellins in groups of three rats/dose level.

The effect of this plant growth stimulant on testicular growth in mammals was ascertained in groups of eight male Holtzman rats, weighing approximately 100 Gm. The animals were medicated orally five days a week for a period of four and one-half weeks with 1 Gm./Kg. and with 0.5 Gm./Kg. of the drug.

Ten per cent aqueous solutions of the drug were applied daily to the backs of male mice, six days a week for three and one-half weeks in order to see whether irritation or histologic changes could be induced.

RESULTS

Intravenous acute toxicity studies in mice showed no deaths at doses as high as 4 Gm./Kg. Orally, doses as high as 15 Gm./Kg. were not lethal. No symptoms were observed. Intraperitoneally, a dose of 2 Gm./Kg. was likewise asymptomatic. There were no delayed deaths.

Subacute toxicity studies, both intravenously for five days at 2 Gm./Kg. and subcutaneously for fourteen days at 1 Gm./Kg., in mice yielded negative findings with respect to both organ toxicity and symptomatology. Four mice on intravenous and four mice on subcutaneous medication were sacrificed for autopsy at the end of the previously described medication periods. The following organs of the above eight animals were sectioned for histopathologic examination: heart, lungs, spleen, liver, kidneys, adrenals, testes, pancreas, stomach, small and large intestines, and bone marrow. None of the organs examined showed any changes which could be attributed to drug action. Ninety-day subacute toxicity studies in both dogs and rats at a dose level of 1 Gm./Kg. showed no changes at autopsy and histological examination[2] which could be attributed to drug action. Both male and female dogs, four male and four female rats from the experimental group as well as two male and two female rats from the control group were sacrificed at the end of the ninety-day period, and the following organs sectioned for histopathologic examination: heart, lungs, thymus, thyroid, aorta, liver, kidneys, spleen, adrenals, gonads (testes, ovary, tube, and uterus), stomach, small and large intestines, pancreas, brain, and bone marrow. The animals gained weight satisfactorily and urinalysis, hematology, function tests, and blood chemistry failed to show any pathologic changes.

During the ninety-day experimental period, the female dog doubled her weight from 4.8 Kg. to 9.7 Kg. The male gained 8.5 Kg. from his initial weight of 5.7 Kg. to his terminal weight of 14.2 Kg. During the medication period, the male consumed 781 Gm. of the drug and the female, 648 Gm.

An additional 10 male and 12 female rats were added to the 1 Gm./Kg. dose and have now been on medication at this level for the past fourteen months. These experiments are still in progress.

Intravenous injections of the drug into three anesthetized dogs at dose levels up to 0.5 Gm./Kg. did not affect either the circulatory or respiratory functions. Intravenous injections of 100 mg./Kg. into two anesthetized cats did not alter the blood pressure response to either serotonin or epinephrine.

Pentothal sodium sleeping times in mice were not altered by gibberellic acid treatment. Oral pretreatment with 1 Gm./Kg. of the drug was likewise ineffective in altering the duration of sleep induced with 100 mg./Kg. of hexobarbital sodium, intraperitoneally, in mice.

The drug showed no anticonvulsant activity in mice at dose levels of 1, 2, and 4 Gm./Kg., orally (4). It likewise did not influence the temperature of either normal rats or animals in which fever had been induced with yeast.

Studies on the effect of gibberellic acid on spontaneous motility of the isolated rabbit ileum showed the plant stimulant to have no effect at 1, 2.5, and 5 mg./ml. bath concentration. The highest concentration used was also without any effect on spasms induced in isolated guinea pig and rabbit ileum by barium (0.32 μg./ml.), acetylcholine (0.05–0.5 μg./ml.), and pilocarpine (0.25–2.0 μg./ml.), and on relaxation induced with atropine (0.01 μg./ml.). Results were likewise negative against histamine (0.01 μg./ml.) in the guinea pig ileum and against epinephrine (0.01 μg./ml.) in the rabbit.

Blood cholesterol and blood glucose levels were essentially negative (4). Total blood plasma cholesterol values were 99 mg. % for those animals medicated with gibberellic acid in their diet at a level of 0.17% as compared with 94 mg. % for control animals, the average per cent blood glucose change, plus its standard deviation, for those animals given 250 and 500 mg./Kg. of the drug were $-1\% \pm 14$ and $-6\% \pm 5$, respectively. Each animal served as its own control. Testicular growth, as measured by weight and histological examination, was not affected. Daily application of the drug onto the skin of mice was also without any effect.

These results, coupled with those of Peck, *et al.* (5), indicate that gibberellic acid is a relatively nontoxic and pharmacologically inert compound when given orally, parenterally, topically, or when tested on isolated organ systems.

SUMMARY

Gibberellic acid was tested by various routes in mice for its acute toxicity and found to be essentially nontoxic. Both subacute toxicity in mice and subchronic toxicity in dogs and rats showed the compound to be asymptomatic and free of pathologic changes, histologically. The highest observed nontoxic (and asymptomatic) doses in mice as determined by acute toxicity testing were as follows: 4 Gm./Kg., intravenously; 15 Gm./

[2] Histopathologic examinations were carried out in the Pathology Section under the direction of Dr. R. J. Stein.

Kg., orally; and 2 Gm./Kg., intraperitoneally. Subacutely, the following doses were tolerated by mice, rats, and dogs for the times indicated: mice, 2 Gm./Kg., intravenously, for five days; mice, 1 Gm./Kg., subcutaneously, for fourteen days; and rats and dogs, 1 Gm./Kg., orally, for ninety days. The following studies were likewise negative: circulation and respiration, barbiturate potentiation, antipyresis, anticonvulsant activity, intestinal motility, blood cholesterol, and blood glucose.

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A Note on the Phytochemical Investigation of *Cecropia peltata* L.*

By NYDIA M. KING and NOELIA HADDOCK

A preliminary investigation of the leaves of *Cecropia peltata* L. has been made. Glycosidal material was obtained from the alcohol extract and also from the aqueous extract. No sterols or alkaloids were found.

Cecropia peltata, L. (*Ambaiba peltata*, Kuntze) commonly known as yagrumo (trumpet tree) is a tall tree with large lobed leaves which are dark green above and silvery-white beneath. It is the type species of the many Cecropias native of the West Indies and continental tropical America (1).

Several therapeutic uses have been claimed for the various parts of the tree. The bark and buds are said to be astringent, the roots and bark have been used as an emenagogue, while the leaves have been employed in acute pulmonary infections, as an antiasthmatic, and as a sedative in nervous conditions such as chorea (2). Grosourdy (2) reports that when using a decoction of the leaves as a sedative the patient experienced a state of passiveness and slowness in all his muscular movements and his heart rate was slowed and made more rhythmical. No cumulative action or side effects, such as digestive tract disorders like those caused by the continuous use of digitalis, were observed. It was found to have a diuretic effect. In view of these observations, yagrumo was thought to be a possible substitute for digitalis (3).

A survey of the literature revealed that very little chemical and pharmacological investigations of *Cecropia peltata* L. have been reported. Valeri and Narváez (4) showed that decoctions and infusions of 15 to 30 Gm. of the leaves per liter were nontoxic to guinea pigs when given by hypodermic injection. They found that the preparations had diuretic and laxative effects in guinea pigs, also causing transitory drowsiness. *In vitro* they had no inhibitory power over pathogenic bacteria of the respiratory apparatus.

Due to its potential use in medicine it seemed desirable to effect a thorough chemical investigation of *Cecropia peltata*, L. The first part of this work is reported here.

* Received June 28, 1958, from the College of Pharmacy, University of Puerto Rico, Rio Piedras.

EXPERIMENTAL

The plant material used in this investigation consisted of the leaves collected locally, dried at 60°, and ground.

Preliminary examination tests, carried out as described by Rosenthaler (5), indicated the presence of tannins, mucins, proteins, and reducing substances while alkaloids and saponins were shown to be absent. According to the Stas-Otto method the material did not contain alkaloids or glycosides. Further examination was made following the general method outlined by Rosenthaler (6).

Sixteen grams of the ground leaves was extracted successively with the solvents mentioned in the order given below. A Soxhlet apparatus was used for all the extractions, except for the extraction with water. The latter was done by maceration in a mechanical shaker and the solvent was removed under reduced pressure. The nonaqueous solvents were allowed to evaporate in air. The values of per cent extractives obtained were as follows: petroleum ether 1.16%, ethyl ether 1.93%, absolute alcohol 6.93%, acetone 3.86%, chloroform 0.84%, and cold water 6.75%.

It was observed that the alcoholic extract deposited rosette-like crystals embedded in dark brown matter. A small amount of the crystalline deposit was readily soluble in water and the aqueous solution gave positive tests for carbohydrates and/or glycosides with a naphthol solution, and also for reducing sugars with Fehling's solution. The noncrystalline portion of the alcoholic extract gave a strong Fehling's test after it had been heated with dilute hydrochloric acid.

The aqueous extract gave positive tests for proteins, alkaloids, and reducing substances. Upon clarification with a solution of lead acetate a residue was obtained which, after boiling with dilute hydrochloric acid, reduced Fehling's solution. Negative results were obtained when the rest of the extracts were tested for the usual substances which might be present.

Except for the deposit formed in the alcoholic extract, the different extracts did not yield any crystalline material, even after prolonged refrigeration.

Twenty grams of the ground leaves was extracted with cold water and a 10% solution of lead acetate was used to clarify the extract. A small amount of a colorless amorphous powder, m. p. 202–205°,[1] was isolated from the lead acetate precipitate by following the usual procedure. Attempts to crystallize this material from acetone, methanol, and a mixture of ethanol and water were not successful, but the melting point changed to 214–215°. The material was insoluble in water, 5% hydrochloric acid, and ether; soluble in 5% sodium hydroxide; less soluble in 5% sodium bicarbonate; soluble in concentrated sulfuric acid with the production of a white precipitate upon dilution with water. It gave a positive Fehling's test after it was boiled with dilute hydrochloric acid.

Crystalline material was obtained from the basic lead acetate precipitate. This material gave a positive Fehling's test, but much more so after boiling with dilute hydrochloric acid. Due to the small amount available it was not possible to investigate it further at the time.

[1] Melting points are uncorrected.

SUMMARY

1. The leaves of *Cecropia peltata*, L. were extracted with various solvents.

2. Impure crystalline material and an amorphous colorless powder, m. p. 214–215°, both giving presumptive tests for glycosides, were isolated.

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A Note on a Simplified Technique for the Administration of Isotopes to Animals*

by RALPH M. LILIENFELD, CHARLES A. ROSS, and ROBERT W. CASE

Quick freezing of a tracer dose of a radioactive isotope provides a simple, precise method of administration to experimental animals and minimizes loss of radioactive substance.

IN ANIMAL ABSORPTION STUDIES using tracer doses, some error inevitably results from loss of the administered isotope which clings to syringe, catheter, and other instruments requisite to the administration. To minimize this loss, we have adopted the following technique: The measured amount of isotope is pipetted into ½ of a gelatin capsule. The capsule is carefully capped and dropped immediately into a test tube containing ethyl alcohol which is immersed in a freezing bath of dry ice and ethyl alcohol. The isotope freezes solid within the gelatin capsule in less than fifteen seconds with no loss of radioactive substance. Prior to its administration, the capsule is removed from the test tube with forceps and exposed to room temperature for a period of thirty seconds. The capsule dissolves within the stomach within five minutes after its oral administration and absorption of the isotope from the G.I. tract is not affected by the freezing process.

* Received July 30, 1958, from the Roswell Park Memorial Institute, Buffalo, N. Y.

A Note on a Method for Thymectomizing the Rat*

By ALFRED P. IACOBUCCI and HOWARD J. JENKINS

THE PROCEDURE for thymectomy in rats has been described in various texts and laboratory manuals. The descriptions of the procedures seem to be lacking in detail, however. It is the purpose of this paper to provide a more complete and detailed description of the surgical technique involved.

In the method reported, male, albino rats of the Carworth-Wistar strain, ranging in weight from thirty-five to fifty grams, are used. Rats of this size are more easily employed since older rats have developed more fatty tissue and heavier fascia. In addition, the thymus glands of smaller rats are larger and more easily removed.

* Received July 22, 1958, from the Edwin Avery Brewer and Howard D. Brewer Pharmacology Laboratories of The Massachusetts College of Pharmacy, Boston.

A procedure reported in a thesis submitted by Alfred P. Iacobucci in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

ANESTHESIA

Anesthesia of moderate depth should be employed. Ether is the ideal anesthetic; but it must be used with extreme caution, otherwise animals may be lost when the deeper planes of anesthesia are reached suddenly and without warning.

In the preliminary work, pentobarbital sodium was administered at the rate of 4 to 5 mg. per 100 Gm. of body weight. Induction with this agent was rapid, but the duration of anesthesia was sufficient to complicate the state of postoperative shock that was encountered. For this reason pentobarbital sodium was considered an unsatisfactory agent for this method.

In other animals, a combination of pentobarbital sodium (3 mg. per 100 Gm. of body weight) and Evipal sodium (6 mg. per 100 Gm. of body weight) administered intraperitoneally was tried. This

TABLE I.—THE EFFECT OF SURGERY TIME AND DURATION OF ANESTHESIA ON MORTALITY RATE[a]

Time, min.: Gland Removal/ Completed Operation	Recovery Time, min. (Pentobarb.)	Mortality, %	Recovery Time, min. (Pentobarb. and Evipal)	Mortality, %	Recovery Time, min. (Ether)	Mortality, %
5/10	120 plus	60	60–90	50	15–30	20
2–5/10	90–120	50	45–90	50	10–15	10
2–5/5–10	90–120	40	45–90	30	10–15	10
2/5	90–120	20	45–90	20	10–15	5

[a] In the above table the time required for the removal of the thymus gland is correlated with the elapsed time of the entire surgical procedure, including closure of the chest cavity; while the recovery time represents the duration of the period between the onset of anesthesia and the regaining of consciousness.

anesthetic mixture shortened the recovery period, and thereby diminished to some extent the possibility of death from postoperative shock complicated by respiratory failure. Even so, some difficulties were evident with this mixture, since Evipal is not well tolerated by young rats.

After considerable experience with its use, ether was selected as the anesthetic for the method. Though some danger of respiratory infection attends its use, recovery is rapid and generally satisfactory.

SURGICAL PROCEDURE

When the rat has been anesthetized, it is placed on its back on an operating board, head toward the operator. The legs are tied to the board and a bit passed behind the incisor teeth and secured in position to hold the head stationary. Hair is removed from the region of the chest and throat. An incision about 2 cm. in length is made along the midline of the thoracic wall from the level of the third rib to a point just beyond the anterior end of the sternum. Here a pointed scissors is inserted under the anterior end and the sternum is cut down to the level of the second rib. (This incision should not exceed 1 cm. in length.) It is essential that the operator work rapidly from this point. Accordingly, the first loop of a continuous suture is started prior to the removal of the gland, so that the chest wall can be closed quickly following extirpation. A size "O," type B, noncapillary thread of silk or nylon attached to a three-quarter inch, half-round, atraumatic, gastrointestinal needle is employed. With blunt probes, the underlying tissue is pushed aside and the chest cavity entered at a point just above the branching of the trachea. The anterior end of the gland is thus exposed. At this point, some respiratory difficulty is exhibited by the rat as a result of the loss of negative pressure in the chest cavity. Both lobes of the gland are held with blunt forceps and a steady even pull is exerted while the gland is gently freed from its surrounding connective tissue. Care is taken to avoid penetrating the great vessels of the heart. When the gland is completely removed, all negative pressure in the chest cavity is lost. Thus, prompt closure of the chest wall is imperative. (It may be necessary to close the incision with a hemostat while suturing.) The cut edges of the thoracic wall are brought into approximation with suture and the skin closed with wound clips. While recovering, the animals exhibit Cheyne-Stokes respiration, but the mortality rate should not exceed 5%. The closed incision is painted with a suitable antiseptic to diminish the possibility of bacterial infection. Postoperative shock is not severe, and the animals begin to gain weight on the day following surgery.

Four or five minutes are required for the entire operation. The actual removal of the gland and closing of the chest cavity should be accomplished within a two-minute period if the mortality rate is to be kept low.

Communications to the Editor

2-(*o*-Tolyl)-cyclohexanol: A Possible Cardioplegic Agent

Sir:

We wish to report that 2-(*o*-tolyl)-cyclohexanol was found capable of suppressing myocardial contraction to the exclusion of significant effect on myocardial electrical properties. During exploratory pharmacological investigations with 2-(*o*-tolyl)-cyclohexanol one of us (G. H. B.) observed that this compound produced reversible mechanical cardiac arrest when perfused as a saturated aqueous solution through isolated cat hearts. The preliminary investigation has been extended to include observations on the isolated rabbit atrium and on the result of intravenous injection in the anesthetized dog. In the isolated preparation, 3 mg. per 100 ml. nutrient solution resulted in virtually complete inhibition of contraction within one minute. With complete mechanical asystole, the transmembrane atrial action potential was essentially unchanged, the membrane resting potential was not altered, and absolute refractory period was not changed. Threshold of electrical excitability increased approximately 10 per cent and stabilized. The

effect on contraction was slowly reversible by washing. In the intact dog, the injection of 60 mg./Kg. (i.v.) resulted in a rapid hypotensive effect leading to complete absence of arterial pressure within one minute. This was presumed to be due in part to depressed ventricular contraction, although it is not known to what extent the drug affects the vasculature directly. In the experiment described, the dog was pharmacologically sympathectomized. No change in spontaneous heart rate, PR interval, QRS duration, or ST duration occurred until many minutes after the onset of the hypotensive effect. Then complete AV block ensued and ventricular conduction slowed. This possibly was due to hypoxia resulting from cardiovascular syncope. Respiration was little affected until agonal gasping occurred.

2-(*o*-Tolyl)-cyclohexanol was prepared from *o*-methylphenyllithium and cyclohexene oxide by the method described by McKusick for the synthesis of the *meta* isomer (1). The product was fractionated through a Todd precise fractionation assembly with a 90-cm. Monel spiral column; b. p. 103–104° at about 1 mm.

Anal.—Calcd. for $C_{13}H_{18}O$: C, 82.07; H, 9.52. Found: C, 81.84; H, 9.27.

The I. R. spectrum of the pure liquid has an intense band at 3,320 cm.$^{-1}$ indicative of hydrogen-bonded hydroxyl groups and a less intense band at 3,450 cm.$^{-1}$ attributed to unassociated hydroxyl groups. The spectrum also has a sharp band at 964 cm.$^{-1}$, a region which falls within the range of characteristic absorption for cyclohexane derivatives (2). The spectra of nine substituted 2-arylcyclohexanols determined at this laboratory (3) all show a band within the range of 962–974 cm.$^{-1}$. The spectra were obtained on a Perkin-Elmer Model 21 spectrophotometer with NaCl prism.

The synthetic method used is expected to give the trans-isomer. This is substantiated by the fact that Cook, Hewett, and Lawrence (4) obtained only one isomer of 2-phenylcyclohexanol from phenyllithium and cyclohexene oxide under similar conditions and that this isomer is now known to be the trans-2-phenylcyclohexanol (5, 6). Investigation of the stereochemistry and of the stereospecificity on the pharmacological properties observed of this and other related compounds is in progress.

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ALAIN C. HUITRIC, THEODORE C. WEST, and ROBERT A. DURBIN
College of Pharmacy, and Dept. of Pharmacology, University of Washington, Seattle 5
GORDON H. BRYAN
School of Pharmacy, Montana State University, Missoula

***Labiatae* Family**

Sir:

Chemical ontogenetic and phylogenetic considerations of the *Labiatae*, relating the occurrence of sterols and certain terpenes, include data (1) on two species of the *Umbelliferae*. Although conclusions were not established concerning the data derived by a perfunctory examination of the unsaponifiable fraction of extracts of these plants, the unqualified presence of the data is misleading. The reader's perception of chemical phylogeny is further inclined by the exclusion of *Marrubium vulgare* L. from the *Labiatae*.

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ARTHUR E. SCHWARTING
University of Connecticut, Storrs

Book Notices

The Interference Microscope in Biological Research. By ARTHUR J. HALE. The Williams & Wilkins Co., Baltimore, exclusive U. S. agents, 1958. xi + 114 pp. 14 x 22 cm. Price $5.

This is an instructive textbook designed to acquaint the biologist with the principles and techniques of interference microscopy. An appendix discusses wave form and polarization of light, and gives examples of calculations. The interference microscope, or microscopic interferometer, is particularly useful in the study of the processes involved in cellular metabolism.

Ciba Foundation Symposium on the Neurological Basis of Behaviour. Edited by G. E. W. WOLSTENHOLME and CECILIA M. O'CONNOR. Little, Brown and Co., Boston, 1958. xi + 400 pp. 13.5 x 20.5 cm. Price $9.

This book presents the papers and discussions of the symposium on the interrelations of neural and behavioral mechanism linking psychology with neurophysiology. The subjects covered range from microphysiology of neuroses to phenomena of behavior and psychology. Analytical, biological, neurophysical, pharmacological, biochemical, clinical, and strictly behavioral methods are considered. A subject index is appended.

Symposium on Protein Structure. Edited by ALBERT NEUBERGER. John Wiley & Sons, Inc., New York, 1958. 351 pp. 15.5 x 23.5 cm. Price $7.75.

This book is a record of the papers and discussions at the symposium on protein structure sponsored by the Protein Commission of the Section of Biological Chemistry of the International Union of Pure and Applied Chemistry held at Paris, July 1957. The subjects are grouped under the headings: General problems and methods, Specific proteins, Proteolytic enzymes, Ribonuclease, Tobacco mosaic virus, Other proteins and peptides. A subject index is appended.

Water and Electrolyte Metabolism in Relation to Age and Sex. Ciba Foundation Colloquia on Ageing, Vol. 4. Edited by G. E. W. WOLSTENHOLME and MAEVE O'CONNOR. Little, Brown and Co., Boston, 1958. xii + 327 pp. 13.5 x 20.5 cm. Price $8.50.

This compilation of the proceedings of the fourth colloquium on ageing covers many problems, including: genetic control of electrolyte metabolism in the erythrocytes, glandular secretion of electrolytes, hormonal aspects of water and electrolyte metabolism in relation to age and sex, the development of acid-base control, severe magnesium deficiency, and the ability of the organism to maintain normal cellular states under various nutritional conditions, the role of the kidney in electrolyte and water regulation in the aged, and age and renal disease. A subject index is appended.

Phosphorus and Its Compounds—In Two Volumes. Vol. I: Chemistry. By JOHN R. VAN WAZER. Interscience Publishers, Inc., New York, 1958. xiii + 954 pp. 15 x 23 cm. Price $27.50.

This is a comprehensive text on phosphorus and its compounds, which is presented with the purpose of laying a foundation for phosphorus chemistry as a new, separate discipline of chemistry. The text is divided under the chapter headings: The phosphorus atom, its nucleus and electronic structure; Interaction between atoms, with especial reference to phosphorus chemistry; Systematic chemistry of phosphorus and its compounds; Elemental phosphorus and the metal phosphides; Hydrides, halides, and pseudohalides of phosphorus and their organic derivatives; Oxides, sulfides, nitrides, and related compounds of phosphorus; Lower oxyacids of phosphorus, their salts and esters; Structure and properties of the condensed phosphates; Orthophosphoric acid, its salts and esters; Individual chain phosphates (pyro-, tripoly-, tetrapoly-, and pentapoly-phosphates as well as Kurrol's and Maddrell's salts); Ring and branched phosphates; Amorphous phosphates, including phosphate glasses, condensed phosphoric acids, and phosphate esters; and Halo-, peroxy-, thio-, and amidoacids of phosphorus, their salts, esters, and related compounds. The text material contains many diagrammatic formulas, illustrations, and tabulated data; and many references are given. Appendixes give a table of 187 phosphate minerals, a collection of single bond energies and distances with electronegativity differences for use in calculations concerning phosphorus compounds, and thermodynamic data on phosphorus compounds. Author and subject indexes are included.

Encyclopedia of Chemistry (Supplement). Edited by GEORGE L. CLARK and G. G. HAWLEY. Reinhold Publishing Corp., New York, 1958. vi + 330 pp. 17.5 x 25.5 cm. Price $10.

This *Supplement*, including more than 200 additional reviews, is stated to be the only volume that will be added to the first volume. The arrangement of the text is the same as in the original volume which was cited in THIS JOURNAL, **46**, 454(1957). The concise discussions in the *Supplement* include: Sedatives and tranquilizers, Sunproofing agents, Drugs (legal aspects of marketing), Antihistamines, Antimalarials, and Dentifrices.

Physico-Chimie Biologique et Médicale. By CHRISTIAN BÉNÉZECH. Masson et Cie., Paris, 1958. vii + 684 pp. 17 x 24 cm. Price 8,000 fr.

This textbook (in French) is intended for biochemists, physiologists, and others in the related fields of medicine. The text is divided into four main parts under the headings: Matter and its transformations, Homogeneous states of living matter (gas-liquid-solution), Dispersed systems and macromolecules, and Elements of physicochemical biology. A subject index is appended.

Radioactive Isotopes in Clinical Practice. By EDITH H. QUIMBY, SERGEI FEITELBERG, and SOLOMON SILVER. Lea & Febiger, Philadelphia, 1958. 451 pp. 15.5 x 23.5 cm. Price $10.

This book describes radioisotopes, tells how they act, and explains their clinical uses in diagnosis and treatment of disease. The text is divided into the three separately authored parts: Basic physics, Instrumentation and laboratory methods, and Clinical applications. The three authors have worked as a team in this field, and their views correlate the information for physicians and technicians. The hazards of radiation and the procedures required to avoid them are stressed. Author and subject indexes are appended. Pharmacists in radioisotope centers will find this a useful reference.

Initiation Mathématique a la Physique Médicale et a la Biologie. By JULIEN GUELFI. Masson et Cie., Paris, 1958. viii + 220 pp. 16.5 x 24 cm. Price 4,000 fr.

This book (in French) is intended as a mathematics text for students of medicine and pharmacy in particular. It ranges from algebraic calculations, elementary geometry, and trigonometry, through calculations of probabilities and statistics.

Disinfection and Sterilization. By G. SYKES. D. Van Nostrand Co., Princeton, 1958. xviii + 396 pp. 15.5 x 25 cm. Price $10.75.

This book, incorporating recent applications and discussions on theory and mode of action of disinfection and methods of sterilization, should be useful to medical bacteriologists, pharmacists, and industrial bacteriologists. The text is divided into six major parts. Part I discusses theory of disinfection and methods of testing disinfectants and antiseptics. Part II covers methods of sterilization. Part III is devoted to air disinfection and sterilization. Part IV discusses disinfection of viruses. Compounds used as disinfectants and antiseptics are taken up in Part V. Part VI deals with methods of preservation in relation to foods, pharmaceutical products, and industrial materials. Author and subject indexes are appended.

Accepted Dental Remedies 1959. 24th ed. American Dental Association, Chicago, 1958. xiv + 210 pp. 15.5 x 20.5 cm. Paperbound. Price $3.

This book contains expanded sections on dental therapeutics. The monographs have been reviewed by the Council and its consultants and have been rewritten in the light of current scientific information. The reorganized format, which made last year's edition so popular, has been retained with minor additional improvements. A discussion of the prescription use of fluoride preparations is included in the chapter on Fluoride Compounds.

As in earlier editions, Accepted Dental Remedies 1959 includes information concerning drugs of recognized value in dentistry, drugs of uncertain status more recently proposed for use by the dentist, and some drugs once employed extensively but now generally regarded as obsolete. Only brands of drugs which the Council has classified in Group A are included in the book.

Guide Pratique de la Laborantine. 3rd ed. By J. MARLIANGEAS. Vigot Freres, Editeurs, Paris, 1958. 144 pp. 16 x 24 cm. Price 1,000 fr.

This book (in French) is intended for beginning laboratory technicians and medical students. It describes techniques and procedures for bacteriologic, hematologic and serologic, histologic, and chemical methods used in the clinical laboratory.

Biochemical Engineering. Edited by R. STEEL. The Macmillan Co., New York, 1958. 328 pp. 14 x 21.5 cm. Price $7.50.

This book is based upon postgraduate lectures on the industrial application of biochemical processes, particularly as they apply to unit processes in fermentation. The text is divided under the chapter headings: Principles of biochemical engineering; Scope of biochemical engineering; Microorganisms and their activities; Substrates for fermentation processes; Sterilization of equipment, Air and media; Development of anaerobic fermentation processes: Acetone-butanol; Oxygen supply and demand in aerobic fermentations; Development of aerobic fermentation processes: Penicillin; Equipment design; Recovery of fermentation products; and Present trends and future developments. A subject index is appended.

Organic Syntheses. Vol. 38. Edited by JOHN C. SHEEHAN. John Wiley & Sons, Inc., New York, 1958. vii + 120 pp. 15 x 23 cm. Price $4.

Methods of synthesis are provided in this volume for the compounds in the list which follows: 2-Amino - 4 - anilino - 6 - (chloromethyl) - s - triazine; 2 - Benzylaminopyridine; Benzyltrimethylammonium ethoxide; 4-Bromo-2-heptene; 2-Bromo-3-methylbenzoic acid; 3-(*o*-Chloroanilino) propionitrile; Dibromoacetonitrile; Dicyclopropyl ketone; Diethyl methylenemalonate; 5,5-Dimethyl-2-*n*-pentyltetrahydrofuran; Diphenylacetaldehyde; N- Ethyl-*p*-chloroanilinine; 5- Formyl - 4 - phenanthroic acid; Hendecanedioic acid; 3-Hydroxytetrahydrofuran; 6-Ketohendecanedioic acid; 2-Methyl-2,5-decanediol; *trans*-2-Methyl-2-dodecenoic acid; 2-Methylenedodecanoic acid; β-Methylglutaric anhydride; Methyl Hydrogen Hendecanedioate; 1-Methylisoquinoline; Methyl *p*-tolyl sulfone; Monobenzalpentaerythritol; Monobromopentaerythritol; Monovinylacetylene; 1-Nitrooctane; 1,4 - Pentadiene; α - Phthalimido - *o* - toluic acid; *trans*-Stilbene oxide; and 2-Vinylthiophene.

Treatment in Internal Medicine. By HAROLD T. HYMAN. J. B. Lippincott Co., Philadelphia, 1958. xiv + 609 pp. 17.5 x 25.5 cm. Price $12.50.

A compendium of current treatment procedures designed to serve as a ready and reliable source of information for the busy physician. Its pharmaceutical interest lies in the discussion and evaluation of many drugs.

Physiology of Spinal Anesthesia. By NICHOLAS M. GREENE. The Williams & Wilkins Co., Baltimore, 1958. xi + 195 pp. 15 x 23 cm. Price $6.

A documented review and evaluation of the physiology of spinal anesthesia.

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